Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Medicine and Health Sciences
Department of Clinical and Molecular Medicine

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Effects of overexpressing NOR-1 on cell death and cell viability in human cardiomyocytes during doxorubicin-induced stress

Master's thesis in Pharmacy
Supervisor: Morten Høydal
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Abstract

Ischemic heart disease (IHD) is the leading cause of death globally, characterized by reduced blood flow to the heart muscle. If left untreated, a complete blockage of the coronary arteries can occur, known as a myocardial infarction (MI). Restoration of the blood flow is critical to prevent irreversible damage to the heart. However, the reperfusion can itself damage the cardiomyocytes (CMs) in the heart because of the excessive formation of reactive oxygen species (ROS), among other things. This damage is called reperfusion injury (RI), and there is currently no optimal treatment to prevent this. Doxorubicin, a widely used anti-cancer drug, is also known to cause cardiotoxicity because of excessive ROS production. Exercise training has been shown to protect the heart against both RI and DOX-induced cardiotoxicity, but the exact mechanism is still unknown. Furthermore, nuclear receptor 4A3 (NR4A3), coding for the protein neuron-derived orphan receptor 1 (NOR-1), is an important exercise- and inactivity-responsive gene in skeletal muscle. Therefore, we hypothesized that NOR-1 could protect CMs against oxidative stress induced by DOX. We also hypothesized that NOR-1 is involved in preparing the CMs against a stress situation during nonstimulated conditions by increasing cell viability. AC16 CMs were transfected with an NR4A3 expression vector and divided into either a DOX treated group, receiving 5 μM of DOX for 12 hours, or a nonstimulated vehicle-treated group. AC16 CMs transfected with an empty vector served as controls in both treatment groups. Lactate dehydrogenase (LDH) activity, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and caspase-3 activity assays were performed to measure cell death, cell viability, and apoptosis, respectively. In addition, western blotting on cell lysate was used to determine expression levels of several proteins known to be involved in cardioprotection. We demonstrated that NOR-1 overexpression decreased cell death (P<0,05) and apoptosis (P<0,01) while increasing cell viability (P<0,05) in DOX-treated AC16 CMs. We also observed that NOR-1 overexpression increased phosphorylation of extracellular signal-regulated kinase (ERK) (P<0,01) and protein expression levels of B cell lymphoma-extra large (Bcl-xL) (P<0,01). We did not measure any significant changes in phosphorylation of protein kinase B (Akt), glycogen synthase kinase-3β (GSK-3β) and signal transducer and activator of transcription 3 (STAT3) or expression levels of superoxidase dismutase 2 (SOD2) and cyclin D1. Furthermore, we demonstrated that NOR-1 overexpression increased the cell viability (P<0,0001) of AC16 CMs during nonstimulated conditions without affecting cell death or apoptosis. No changes in expression levels of previously mentioned proteins were observed after NOR-1 overexpression. Thus, our findings indicate that NOR-1 could serve as a potential cardioprotective protein in response to cellular stress.

Sammendrag

Koronar hjertesykdom er den ledende dødsårsaken i verden, karakterisert av redusert blodforsyning til hjertet. Ubehandlet kan dette føre til en fullstendig blokkering av koronararteriene, kjent som et hjerteinfarkt. Gjenåpning av den blokkerte arterien er kritisk for å unngå irreversibel skade på hjertet. Paradoksalt nok, så kan reperfusjon i seg selv skade kardiomyocyttene i hjertet på grunn av blant annet kraftig økning i reaktive oksygenforbindelser. Denne skaden kalles reperfusjonsskade og det finnes per i dag ingen optimale behandlingsmetoder for å unngå dette. Doksorubicin (DOX), en ofte brukt kreftmedisin, er også kjent for å være toksisk for kardiomyocytter på grunn av økt produksjon av reaktive oksygenforbindelser. Trening kan beskytte hjertet mot både reperfusjonsskade og DOX-indusert hjerteskade, men den eksakte mekanismen er fortsatt ukjent. Videre så er det vist at nuclear receptor 4A3 (NR4A3), som koder for proteinet neuron-derived orphan receptor 1 (NOR-1), er det genet som responderer mest på trening og inaktivitet i skjelettmuskulatur. Vår hypotese var derfor at NOR-1 kunne beskytte kardiomyocytter mot oksidativt stress forårsaket av DOX. I tillegg var hypotesen vår at NOR-1 kunne forberede kardiomyocyttene mot en eventuell stress-situasjon i ustimulerte forhold ved å øke celleviabiliteten. AC16 kardiomyocytter ble transfektert med en vektor som inneholdt NR4A3 genet for å overuttrykke NOR-1. Kardiomyocyttene ble så delt inn i enten en DOX-behandlet gruppe, som ble utsatt for 5 µM DOX i 12 timer, eller en ustimulert kontrollgruppe. AC16 kardiomyocytter transfektert med en tom vektor fungerte som en kontroll i begge gruppene. Laktatdehydrogenase (LDH)-, 3-(4,5-dimetyltiazol-2-yl)-2,5-difenyltetrazoliumbromid (MTT)- og caspase-3 aktivitetsanalyser ble utført for å måle henholdsvis celledød, celleviabilitet og apoptose. I tillegg ble western blot utført for å måle utrykket av forskjellige proteiner som er kjent for å være hjertebeskyttende. I denne studien viste vi at overuttrykk av NOR-1 reduserte celledød ($P<0,05$) og apoptose ($P<0,01$), mens celleviabiliteten økte ($P<0,05$) i DOX-behandlede AC16 kardiomyocytter. Vi observerte i tillegg en økning i fosforyleringen av ekstracellulær signal-regulert kinase (ERK) ($P<0,01$) og økt proteinuttrykk av B cell lymfoma-extra large (Bcl-xL) ($P<0,01$). Vi så ingen signifikant endring i fosforyleringen av protein kinase B (Akt), glykogen syntase kinase-3β (GSK-3β) og signal transducer and activator of transcription 3 (STAT3), eller proteinutrykket av superoksid dismutase 2 (SOD2) og syklin 1. Under ustimulerte forhold, så førte overuttrykk av NOR-1 til økt celleviabilitet ($P<0,0001$), uten å påvirke celledød eller apoptose. Ingen signifikante endringer i de tidligere nevnte proteinene ble observert. Våre funn kan tyde på at NOR-1 beskytter hjerteceller i respons til stress.

Acknowledgments

This master thesis is a part of the two-year study program Master of Science in Pharmacy at the Norwegian University of Science and Technology (NTNU). The work in this project was carried out at the Department of Circulation and Medical Imaging, a part of the Faculty of Medicine and Health Sciences. My supervisor has been Associate Professor Dr. Morten Høydal, and my co-supervisor has been Dr. Gurdeep Marwarha.

Many thanks to Morten for the excellent guidance and constructive advice you have given me throughout this project. Your availability and positivity have been of utmost importance this year. The continuous feedback you were able to provide during the writing of this thesis is remarkable. This project has been a great learning experience and offered a valuable insight into the world of science.

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Lastly, I want to thank my girlfriend for supporting me throughout this master thesis and motivating me during the writing process.

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Abbreviations

Apaf-1	apoptotic protease activating factor 1
ATP	adenosine triphosphate
Akt	protein kinase B
Bad	Bcl-2-associated death promoter
BAG	Bcl-2 interacting protein
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated protein X
Bcl-2	B cell lymphoma-2
Bcl-xL	Bcl-extra large
Bid	BH3 interacting-domain death agonist
Bik	Bcl-2-interacting killer
Bim	Bcl-2-like protein 11
BH3	Bcl-2 homology 3
BSA	bovine serum albumin
cIAP2	cellular inhibitor of apoptosis 2
CMs	cardiomyocytes
CVD	cardiovascular diseases
DOX	doxorubicin
DMEM	Dulbecco's Modified Eagle's Medium
ERK	extracellular signal-regulated kinase
ETC	electron transport chain
EV	empty vector
FBS	fetal bovine serum
GSK-3β	glycogen synthase kinase-3β
HRP	horseradish peroxide
HSP	heat shock protein
IAP	inhibitors of apoptosis
IHD	ischemic heart disease
IPC	ischemic preconditioning
iPS	induced pluripotent stem cells
JAK	Janus kinase
LDH	lactate dehydrogenase
NOR-1	neuron-derived orphan receptor 1
Noxa	phorbol-12-myristate-13-acetate-induced protein 1
NR4A3	nuclear receptor 4A3
MAC	mitochondrial apoptosis-induced channel
MI	myocardial infarction
mPTP	mitochondrial permeability transition pores
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC	nitrocellulose
PBS	phosphate-buffered saline
PCI	percutaneous coronary intervention
Puma	p53 upregulated modulator of apoptosis
PVDF	polyvinylidene fluoride
RISK	reperfusion injury salvage kinase
ROS	reactive oxygen species
RI	reperfusion injury
RSK	ribosomal S6 kinase
SAFE	survivor activating factor enhancement
Ser	serine
SDS	sodium dodecyl sulfate

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	superoxidase dismutase
STAT	signal transducer and activator of transcription
STEMI	ST-elevation myocardial infarction
TBS-T	tris buffered saline-Tween 20
TNF-α	tumor necrosis factor α
Top2	topoisomerase II
TRAIL	TNF-related apoptosis-inducing ligand
TWEAK	TNF-related weak inducer of apoptosis
VSMC	vascular smooth muscle cells
WHO	World Health Organization

1. Introduction

1.1. Ischemic heart disease

Ischemic heart disease (IHD) is the leading cause of death worldwide. 8,9 million people died of IHD in 2019, and this disease has the most significant increase in fatalities over the past few years, according to World Health Organization (WHO) (1). IHD occurs when the blood supply to the heart is inadequate due to a narrowing or complete blockage of the blood vessels. Narrowing of the blood vessels is most often caused by atherosclerosis, which is a buildup of plaque in the lining of the artery wall (2). If left untreated, plaque buildup can rupture and cause the formation of a blood clot that completely blocks the coronary artery (Fig. 1). This abrupt cutoff of blood supply is called a myocardial infarction (MI) (3). A less common cause of MI is coronary artery spasms, where there is a severe spasm in the coronary artery, resulting in a cutoff of the blood flow (4).

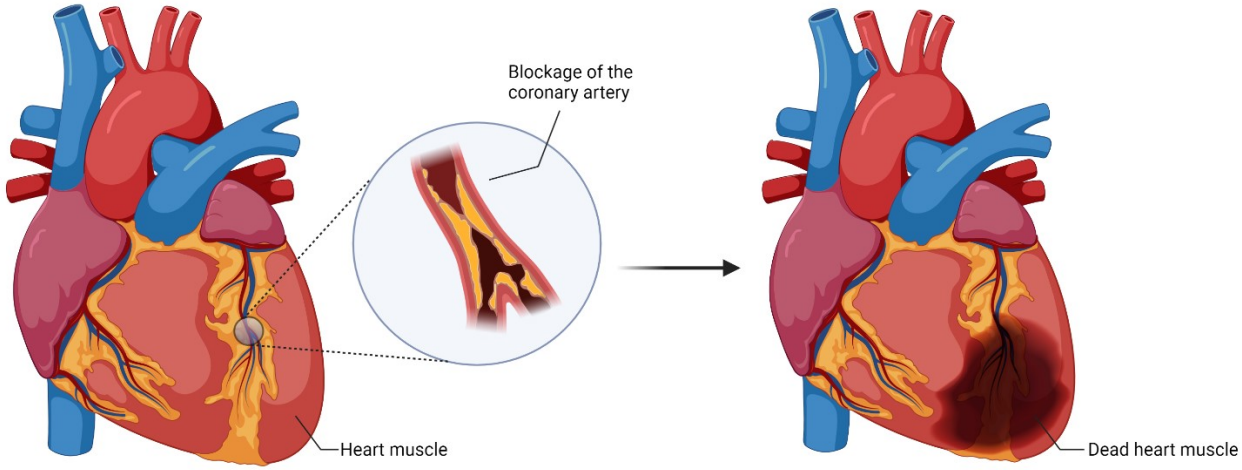

Figure 1: Myocardial infarction. Narrowing of the coronary arteries, most often caused by a buildup of atherosclerotic plaque, leads to inadequate oxygenation of the myocardium. This buildup of plaque can rupture and result in a blood clot that completely blocks the coronary artery. Myocardial cell death follows as a result of insufficient oxygen delivery (3). Created with BioRender.com.

Immediately after the coronary artery gets blocked, a series of severe biochemical and metabolic changes occur within the myocardium because of the deprivation of oxygen and nutrients. First, the absence of oxygen leads to a cellular shift from mitochondrial oxidative phosphorylation to anaerobic glycolysis, which causes adenosine triphosphate (ATP) depletion and a buildup of lactate and H^+. The buildup of H^+ then causes the activation of the Na^+/H^+ ion exchanger, which extrudes H^+ in exchange for Na^+ entry. ATP depletion causes a reduction in Na^+/K^+ ATPase activity, which further increases the intracellular Na^+. The Na^+/Ca^{2+} ion exchanger acts in reverse to combat this, but this results in intracellular Ca^{2+} overload (5). Following this, the accumulation of H^+, Na^+, and Ca^{2+} lead to hyperosmolarity, resulting in water influx and cell swelling. Decreased cellular pH, caused by the buildup of H^+, impairs the enzyme activity in the cells and leads to clumping of nuclear chromatin (6). In addition, due to reduced coronary flow, the metabolic waste is not removed and can interfere with ATP production (7). If left untreated, irreversible cell damage occurs by cells rupturing due to physical defects in the cell membrane (8).

Treatment is time-critical due to the irreversible cell death occurring after an MI. The main goal is to remove the blockage so that the myocardium can be reperfused and reoxygenated to prevent further damage (3). Percutaneous coronary intervention (PCI) is the most common way to open a blocked coronary artery. A small lead catheter is inserted in either the femoral or radial artery during PCI and guided through the blocked coronary artery. Next, a balloon gets attached to the lead catheter and directed to the blocked artery. With the balloon in place, it inflates and pushes the artery open. A stent is placed around the balloon in most cases, preventing the artery from collapsing again after removing the balloon (9). Primary PCI is the recommended reperfusion strategy to treat a patient diagnosed with ST-elevation MI (STEMI) and should be performed within 120 minutes after the diagnosis. If primary PCI isn't possible within 120 minutes, a fibrinolytic drug should be administered to the person 10 minutes after confirmed STEMI diagnosis and then taken to a hospital for PCI (10).

1.2. Reperfusion injury

Minimizing the ischemic time by early reperfusion is the most crucial factor in reducing the extent of ischemic injury (11). Paradoxically, reperfusion of the myocardial tissue can damage the ischemic tissue and cause the death of cardiomyocytes (CMs). This damage is called myocardial reperfusion injury (RI), and there is currently no effective treatment to prevent this (12). Restoration of the blood flow allows the mitochondrial membrane potential to recover, which leads to further mitochondrial Ca^{2+} overload. During ischemia, the mitochondrial permeability transition pores (mPTP) remain closed due to the low pH. However, the increase in pH during reperfusion, combined with the mitochondrial Ca^{2+} overload, results in the opening of mPTP. Prolonged opening of mPTP results in irreversible damage during reperfusion (13).

Another critical factor of RI is thought to be the excess production of reactive oxygen species (ROS) during and following reperfusion. ROS are chemical compounds derived from molecular oxygen, which has acquired less than 4 electrons. Some examples are the superoxide anion radical, hydrogen peroxide, and hydroxyl radical (14). Mitochondria, NADPH oxidase, xanthine oxidase, and uncoupled nitric oxide synthase are the main contributors to ROS production during reperfusion, but how much they contribute varies based on the tissue (15). In the heart, the main contributors are thought to be the mitochondria and NADPH oxidases (16). Mitochondrial ROS production is complicated and still not fully understood. One theory is that defects in the electron transport chain (ETC) caused by ischemia result in increased ROS production during reperfusion. Excessive mitochondrial ROS production can also lead to the opening of mPTP (17). Furthermore, reperfusion of the myocardium results in cells releasing chemical mediators that activate NADPH oxidases, which also leads to increased ROS production (6). In addition to mPTP opening, ROS can also induce damage to proteins, nucleic acids, and lipids (17).

Most cells have protective mechanisms to protect against ROS, which include both non-enzymatic and enzymatic antioxidant systems. Superoxidase dismutase (SOD) is an enzyme family which converts superoxide anions to hydrogen peroxide. The enzymes catalase, glutathione peroxidase, and peroxiredoxin then convert the hydrogen peroxide into water and oxygen (18). ROS production during reperfusion overwhelms the endogenous antioxidant system (19), leading to apoptosis and necrosis of the myocardial tissue (20).

Apoptosis can be divided into the intrinsic and extrinsic apoptotic pathways. Activation of the intrinsic pathway involves regulating pro-apoptotic and anti-apoptotic members of the B cell lymphoma-2 (Bcl-2) family. Bcl-2-associated protein X (Bax), Bcl-2 homologous antagonist/killer (Bak), BH3 interacting-domain death agonist (Bid), Bcl-2-like protein 11 (Bim), p53 upregulated modulator of apoptosis (Puma), phorbol-12-myristate-13-acetate-induced protein 1 (Noxa), Bcl-2-associated death promoter (BAD), and Bcl-2-interacting killer (Bik) are the pro-apoptotic members, and Bcl-2, Bcl-x, Bcl-extra large (Bcl-xL), and Bcl-2 interacting protein (BAG) are the anti-apoptotic members (21). The intrinsic pathway is triggered by internal stress, such as DNA damage, ROS, hypoxia, and heat shock, that can activate the Bcl-2 homology 3 (BH3)-only proteins Bid, Bim, Bik, Bad, Puma, and Noxa. These inhibit the anti-apoptotic Bcl-2 members and activates Bax and Bak (22). Bax and Bak activation form a mitochondrial apoptosis-induced channel (MAC) in the outer mitochondrial membrane, releasing intermembrane cytochrome c to the cytosol (23). Opening the mPTP is another way for the mitochondria to release cytochrome c, even though mPTP opening seems to be more involved in necrosis and late apoptosis (24). The opening of mPTP is caused by mitochondrial ROS and calcium overload (17), but studies have shown that the opening of mPTP is dependent on Bax and Bak (25). Following cytochrome c release, it interacts with apoptotic protease activating factor 1 (Apaf-1) to form an apoptosome complex, triggering caspase-9 to activate caspase-3 (26). Suppression of caspase-3 is reported to inhibit apoptosis, making it a crucial protein for the apoptotic pathway (27). The anti-apoptotic members of the Bcl-2 family can prevent apoptosis, but the inhibitors of apoptosis (IAP) family are examples of other proteins that also can prevent apoptosis by inhibiting the activation of several caspases (28).

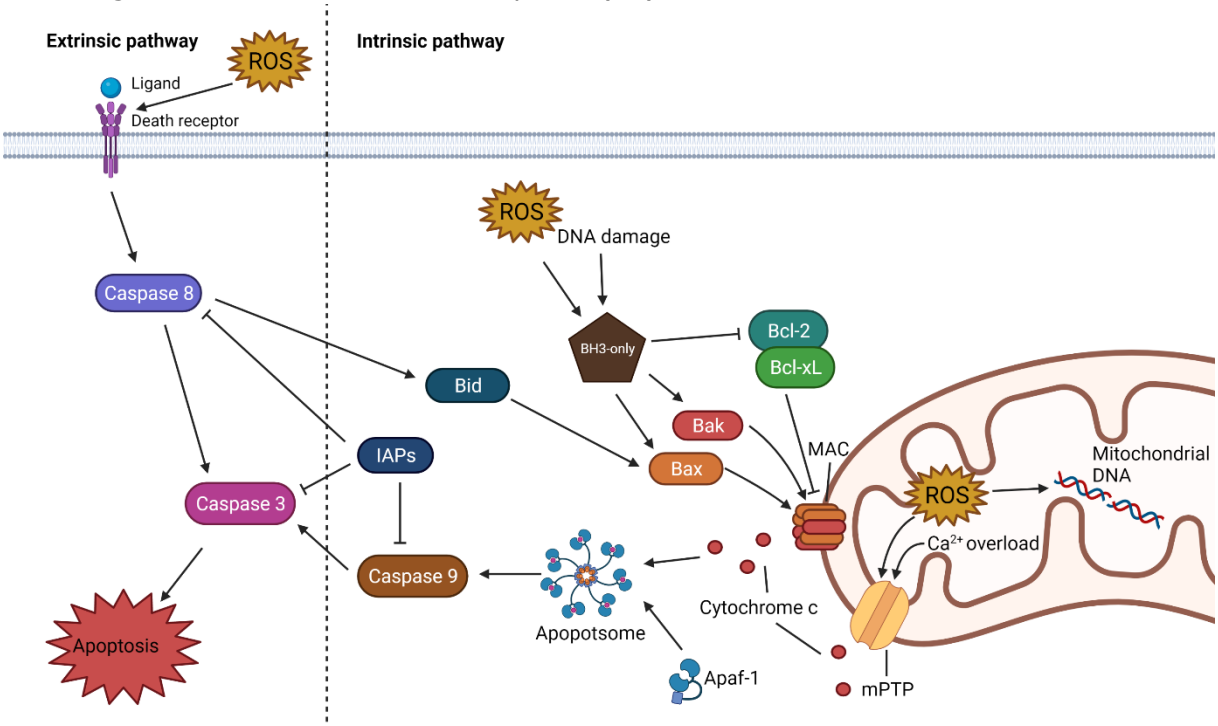

Figure 2: Simplified overview of the extrinsic and intrinsic apoptotic pathway. Molecular mechanisms on how activation of the intrinsic and extrinsic apoptotic pathway leads to apoptosis and the involvement of ROS (21, 22). ROS, reactive oxygen species; IAPs, inhibitors of apoptosis; Apaf-1, apoptotic protease activating factor 1; mPTP, mitochondrial permeability transition pores; Bcl-2, B cell lymphoma-2; Bid, BH3 interacting-domain death agonist; Bax, Bcl-2-associated protein X; Bak, Bcl-2 homologous antagonist/killer; BH3-only, Bcl-2 homology 3-only; Bcl-xL, B cell lymphoma-extra large; MAC, mitochondrial apoptosis-induced channel. Created with BioRender.com

Extrinsic pathway activation is primarily mediated through interaction between ligands, for example, tumor necrosis factor α (TNF-α), TNF-related weak inducer of apoptosis (TWEAK), TNF-related apoptosis-inducing ligand (TRAIL), Fas ligand, and death receptors in the cell membrane (21). ROS is also known to be involved in activating various death receptors (22). This interaction results in the activation of caspase-8, which activates caspase-3 and terminating the cell the same way as the intrinsic pathway. Caspase-8 can also induce the pro-apoptotic Bcl-2 member Bid, triggering the intrinsic pathway and cytochrome c release from the mitochondria (21).

Apoptosis is a form of controlled cell death, leading to limited amounts of inflammation. In contrast, necrosis is an uncontrolled, passive form of cell death. When the cell experience so much excessive external stress (hypoxia, heat, ROS, etc.) that it cannot function, the cell membrane rupture and release its content to the surrounding areas. This release of cellular components causes inflammation and tissue damage in the area (6).

One of the strategies that have been proposed to protect the heart against RI is ischemic preconditioning (IPC). Short periods of ischemia before a major ischemic period protects the heart during an MI (29), and phosphorylation of the pro-survival kinases protein kinase B (Akt) and extracellular signal-regulated kinase (ERK) seem to be involved (30). These two proteins are part of the reperfusion injury salvage kinase (RISK) pathway (31). However, acute activation of the RISK pathway is of the essence since chronic activation of Akt leads to cardiac hypertrophy, and this is also the case for ERK (32, 33). Phosphorylation of Akt during IPC may lead to cardioprotective effects via inhibition of Bad, Bax, and glycogen synthase kinase-3β (GSK-3β), which in the end results in inhibition of mPTP opening (34). Activation of ERK has also been shown to inhibit BAD via p90 ribosomal S6 kinase (RSK). BAD is inhibited by phosphorylation at serine (Ser) 112 by p90RSK, which results in loss of its antagonistic effects over the anti-apoptotic proteins Bcl-2 and Bcl-xL (35). ERK can also inhibit GSK-3β by priming it for phosphorylation at Ser9 by p90RSK (36). Studies have shown that the inactivation of GSK-3β plays a crucial role in myocardial survival in RI (37).

Apart from activating the RISK pathway, IPC can also activate the survivor activating factor enhancement (SAFE) pathway. This pathway involves TNF-α, Janus kinase (JAK), and signal transducer and activator of transcription (STAT) 3 (31). Activation of this pathway results in STAT3 translocating to the mitochondria and inhibiting mPTP opening (38). However, some studies suggest that STAT5, and not STAT3, is responsible for the myocardial protection of IPC in humans (39, 40).

Massive amounts of ROS generation are lethal to CMs. However, it has now been proposed that a small amount of ROS production plays an essential role in IPC (41). In the early phase of IPC, ROS can activate the pro-survival kinases involved in the RISK pathway. Also, ROS can, in the late stage of IPC, activate the SAFE pathway (42).

Since early revascularization still is the most effective way of treating MI, preconditioning the heart will delay the initiation of revascularization, which diminishes the effect of early revascularization (43). Other methods of preparing the heart for an MI and protect against RI are therefore of interest.

1.3. Exercise training

Physical activity and exercise training have proven to reduce the risk of IHD and cardiovascular diseases (CVD). Some of the potential benefits of exercise include reduced blood pressure, decreased myocardial oxygen demand, and increased mitochondrial density, to name a few (44). This means that exercise training reduces the risk of having an MI, but exercise training has also been shown to protect the myocardium against RI (45-47). The exact mechanism which provides this protection is not well understood, but there are some similarities between exercise training and IPC. Long-term aerobic exercise has been shown to protect the heart against RI by increased phosphorylation of Akt and inhibitory phosphorylation of GSK-3β (48). Exercise training has also been shown to activate JAK2/STAT3 signaling pathway in rats, protecting the heart against ischemic injury (49). During intense exercise training, the formation of ROS can lead to oxidative damage to cell components. However, regular exercise training can elevate ROS production to a tolerable level of damage, which in turn upregulates the cellular antioxidant systems (50).

Heat shock protein (HSP) 72 and SOD have both received attention as possible factors which give the cardioprotective effect from exercise training (51, 52). Exercise training increases cardiac HSP72 levels, and overexpression of HSP72 in mice has been shown to protect the heart against RI (53, 54). However, studies have found that increased myocardial HSP72 is not a requirement for cardioprotection after exercise (55, 56). SOD is, as mentioned earlier, an antioxidant family of enzymes that control cellular levels of ROS. SOD consists of three isozymes which are the cytosolic copper/zink-containing variant (CuZn-SOD/SOD1), the mitochondrial manganese variant (Mn-SOD/SOD2), and the extracellular variant (EC-SOD/SOD3) (57). The increase in SOD after exercise training would augment the cell's capacity to scavenge ROS and could be a contributor to the protection against IR in exercise-trained animals (58). However, an increase in enzyme activity and expression of SOD2 has only been observed in studies with more extended periods of exercise training (59, 60) and not in acute training (61). Much work has been done to understand the cardioprotective effects of exercise training, but the exact cellular mechanisms responsible remain unclear (62). Less explored targets might be responsible for the cardioprotective effects seen after exercise training.

1.4. Neuron-derived orphan receptor 1

A meta-analysis with data from 13 studies of resistance-based exercise training, 11 studies of aerobic-based exercise training, 12 studies of acute aerobic exercise, and 8 studies of acute resistance exercise identified nuclear receptor 4A3 (*NR4A3*) as one of the most exercise- and inactivity-responsive gene in skeletal muscle (63). As an early response gene, *NR4A3* is induced by various physiological stimuli, including growth factors, cytokines, and hormones (64). Neuron-derived orphan receptor 1 (NOR-1), encoded by the *NR4A3* gene, is a member of the nuclear receptor family of intracellular transcription factors and is considered to be ligand-independent and constitutively active. NOR-1 regulates transcriptional activity mainly by expression levels, protein-protein interactions, and posttranslational modifications (65).

There are to this day not many studies on the effect of NOR-1 in CMs. One study found that overexpression of NOR-1 in rat hearts increased the activity of JAK2/STAT3 after an MI, which resulted in reduced infarct size, neutrophil infiltration, and pro-inflammatory

cytokine release (66). Meanwhile, in neonatal rat CMs, NOR-1 overexpression led to exacerbated hypertrophy after isoprenaline treatment (67).

In skeletal muscle, NOR-1 overexpression led to increased mitochondrial DNA copy number and improved oxidative capacity (68). Also, NOR-1 directly increases lipin 1 expression, leading to increased cellular capacity for oxidative metabolism (69). Lipin 1 has been found to regulate the cytosolic activation of ERK in skeletal muscle (70).

An interesting finding in vascular smooth muscle cells (VSMC) is that NOR-1 is involved in the survival response during hypoxia. NOR-1 increases cellular inhibitor of apoptosis 2 (cIAP2) expression, which is a member of the IAP family with the ability to inhibit apoptosis (71).

The function of NOR-1 is different between the tissues in the body and sometimes expresses opposite functions based on the tissue. For example, in hepatocytes, NOR-1 promotes proliferation (72). In contrast, NOR-1 promotes apoptosis in several cancer lines by activating Puma and Bax (73). Herring et al. published a review of the functions of the NR4A family on proliferation, apoptosis, and fuel utilization across tissues (74). Most of the processes involving NOR-1 are visualized in figure 3. The function of NOR-1 in CMs during normal and pathological states is still poorly understood, and further research is needed to explore the potential benefits or downsides of NOR-1.

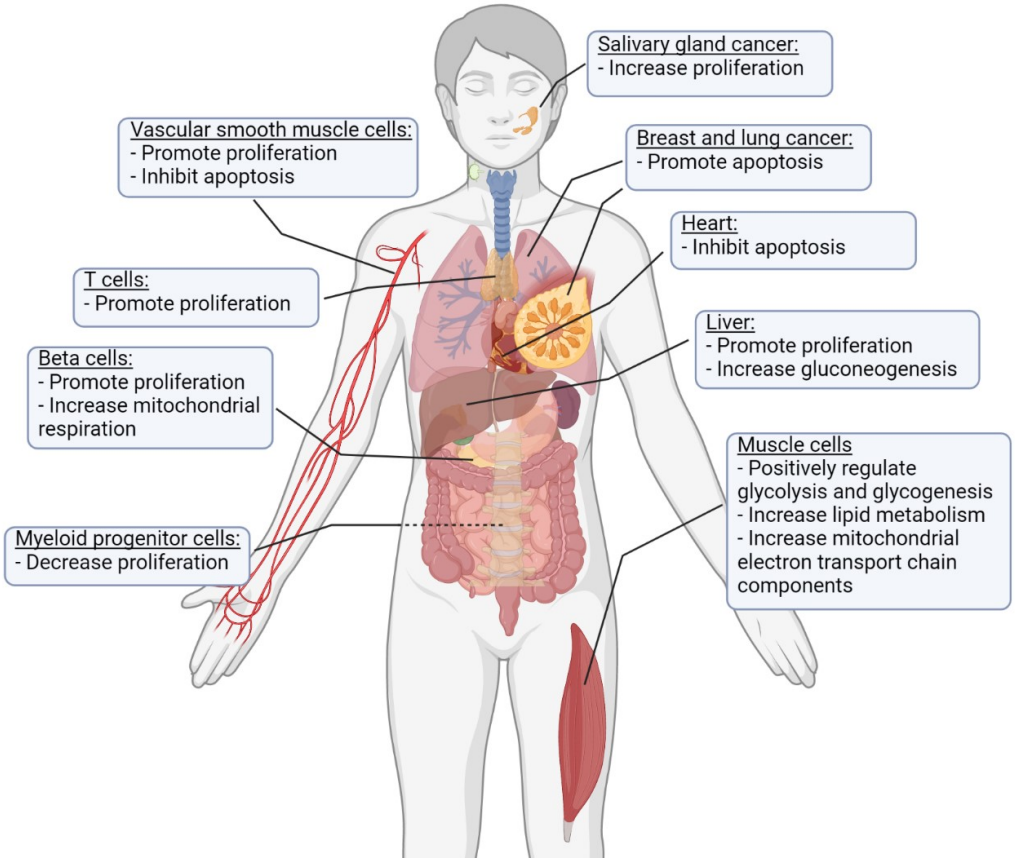

Figure 3: Involvement of NOR-1 in different types of tissue. The figure shows the involvement of NOR-1 in various tissues and cell types across the body based on the review by Herring et al. (74). One thing to note is that some of the functions of NOR-1 are only observed in animals or cell lines. Created with BioRender.com.

1.5. Doxorubicin-induced cardiotoxicity

Doxorubicin (DOX) is a widely used anti-cancer drug used against different types of cancers such as carcinomas, sarcomas, and hematological cancers (75). However, the use of DOX in cancer treatment has severe and sometimes lethal side effects. The most feared side effect is the development of both short-term and long-term cardiotoxicity (76). Acute cardiotoxicity occurs within 2-3 days after administration of DOX and is not dose-dependent. With appropriate treatment, the cardiotoxicity is reversible. On the other hand, chronic cardiotoxicity is related to the dose of DOX administered and may occur as late as ten years after administration (77). There are currently no effective strategies to reduce the risk of DOX-induced cardiotoxicity.

The primary mechanism of DOX-induced cardiotoxicity is increased oxidative stress caused by ROS production and lipid peroxidation (78-80). DOX accumulates in the inner membrane of the mitochondria by binding to the abundant phospholipid cardiolipin (81). This accumulation results in disruption of the ETC and ROS formation (82). Also, NADPH oxidase is reported to be involved in ROS production after DOX administration (83). Other mechanisms in which DOX induce cardiotoxicity are suggested to be inhibition of nucleic acid and protein synthesis, lysosomal changes, and release of vasoactive amines, to name a few (84). Furthermore, a study on induced pluripotent stem cells (iPS)-derived CMs treated with DOX resulted in the upregulation of death receptors, leading to apoptosis (85).

The mechanism responsible for the cardiotoxicity seems different from DOX's anti-cancer activity (84). In cancers, DOX inhibits topoisomerase II (Top2)α, but Top2α is undetectable in heart tissue (85). This difference raises the hope of finding a method to prevent the cardiotoxic effects of DOX without altering its anti-cancer properties. Some studies have shown that exercise training attenuates DOX-induced cardiotoxicity (86-88). One of these studies suggested that the increase in HSP and SOD after exercise training could be responsible for the cardioprotective effect (86). As mentioned earlier, the mechanism responsible for the cardioprotective effect of exercise training during RI is not fully understood. This lack of knowledge raises the question of whether exercise training could protect the heart against DOX-induced cardiotoxicity via similar mechanisms and if NOR-1 is involved.

2. Aim

The main aim of this project was to investigate the effect of NOR-1 overexpression in CMs. This study was divided into two parts ran in parallel. The first part was to observe the impact NOR-1 overexpression elicits in CMs during nonstimulated normal medium conditions. The second part was to observe the effects of NOR-1 overexpression on CMs during an induced oxidative stress condition. This project was then divided into two main objectives:

1. **Investigate if NOR-1 overexpression alters cell death and cell viability in CMs.**
   Comparing the results from cell death and cell viability assays between AC16 CMs treated with NOR-1 overexpression versus nontreated CMs could give insight into the potential benefits of NOR-1. This task was performed both in nonstimulated conditions and in an oxidative stress condition induced by DOX.
2. **Investigate signaling pathways to support potential alterations on cell death and cell viability induced by NOR-1 overexpression.**
   Investigating signaling pathways by measuring protein expression in the cells could give insight into the potential cardioprotective effects of NOR-1 against DOX-induced stress. Furthermore, exploring if NOR-1 overexpression alters protein expression during nonstimulated conditions could provide insight into the function of NOR-1 in CMs not exposed to stress.

The hypotheses for this study were as follows:

1. **NOR-1 overexpression protects the CMs during DOX-induced stress against cell death.**
2. **NOR-1 overexpression prepares the CMs during nonstimulated conditions against potential stress by increasing cell viability.**

3. Methods and Materials

All reagents, with the place of origin and catalog numbers, are listed in appendix 1.

3.1. Cells and cell treatment

In this study, an immortalized proliferating cell line named AC16 will be used to study the regulation of CMs in a normal and pathological state. AC16 CMs are derived from adult ventricular heart tissue fused with a fibroblast cell line. Both nuclear DNA and mitochondrial DNA are retained from the primary CMs, which provides a good in vitro model to explore signaling pathways and address questions of cardiac biology at the cellular level (89). There are, however, certain limitations with using cell lines. The cell lines are studied without interactions from other cell types found in their original local environment. Also, cell lines do not entirely mimic the primary cells, so caution should be taken when drawing conclusions (90). For example, AC16 CMs lack contractile activity because they do not contain sarcomeres (89). The AC16 CMs do, however, yield high biological sample volume and are easily genetically manipulated.

AC16 CMs (Millipore) were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco), containing 12,5% fetal bovine serum (FBS; Gibco) and 1% Antibiotic Antimycotic Solution (Sigma). DMEM with FBS and Antibiotic Antimycotic Solution is from now on referred to as culture medium. AC16 CMs stored in liquid nitrogen were thawed and subcultured into multiple cell culture plates (100 mm diameter). Before the transfection step, the passage of the cells was between 6 and 9. The cells were incubated in a humid atmosphere at 37°C with 5% CO_2.

3.1.1. Transfection of AC16 CMs

Transfection is a way to study the function of genes and gene products in a cell. The way transfection work is by introducing DNA or RNA into the cells by either biological, chemical, or physical treatment. Cells can undergo a stable transfection, which means that the transfected material is incorporated into the host genome and thereby passed on to daughter cells. The other type of transfection is transient transfection. In this case, the foreign DNA is delivered into the cells but not incorporated into the genome. A biological method to transfect cells is to use a virus to transfer the nucleic acids into the cells (91). This process is also known as transduction and can be transient if adenovirus is used or stable if lentivirus is used (92). Even though transduction is an effective and easy way to transfect cells, it can incorporate at a place in the genome where it causes interruption of essential genes if lentivirus is used. Another drawback with using transduction is the potential hazard to laboratory personnel (91).

In this study, a chemical method of transfecting was used. Positively charged cationic lipids complexes with the negatively charged DNA and these net positively charged complexes are thought to enter the negatively charged cell membrane through endocytosis (93). This method is safer than using transduction, but several factors influence the effectiveness. The pH, cell membrane conditions, and amount of cationic lipids used can all affect the efficiency (91, 94). By using this chemical method of transfection, the DNA is not incorporated into the genome, which means that the gene of interest is only translated during the transfected cell's cycle and not in daughter cells.

The AC16 CMs were transfected with either pReceiver-M12 expression-vector (GeneCopoeia) containing the *NR4A3* gene or its corresponding empty vector (EV) using

PolyFect Transfection Reagent (QIAGEN). The expression vector for NR4A3 was tagged at the N-terminus with a 3xFLAG peptide for verification by western blotting.

Before the transfection, a transfection mix for the NR4A3 vector and EV was made. This was made by mixing the expression vector with serum- and antibiotic-free DMEM before waiting 5 minutes and adding the PolyFect Transfection Reagent. The amount of DMEM added was 50 times the amount of vector used, and the amount of transfection reagent was five times the amount of vector. This mix was incubated for 30 minutes at 37°C. The cells of 12 confluent plates of AC16 CMs were collected in two separate tubes and spun down. The two pellets formed were added to either the NR4A3 vector transfection mix or the EV transfection mix and plated into a total of 16 new plates. Each plate now contained 900 ng of expression-vector. 5 mL of culture medium was added to each plate before incubating for six hours at 37°C. Figure 4 shows the different steps in the transfection. After the six hours incubation, the condition-medium was replaced with fresh culture medium and incubated for 24 hours at 37°C.

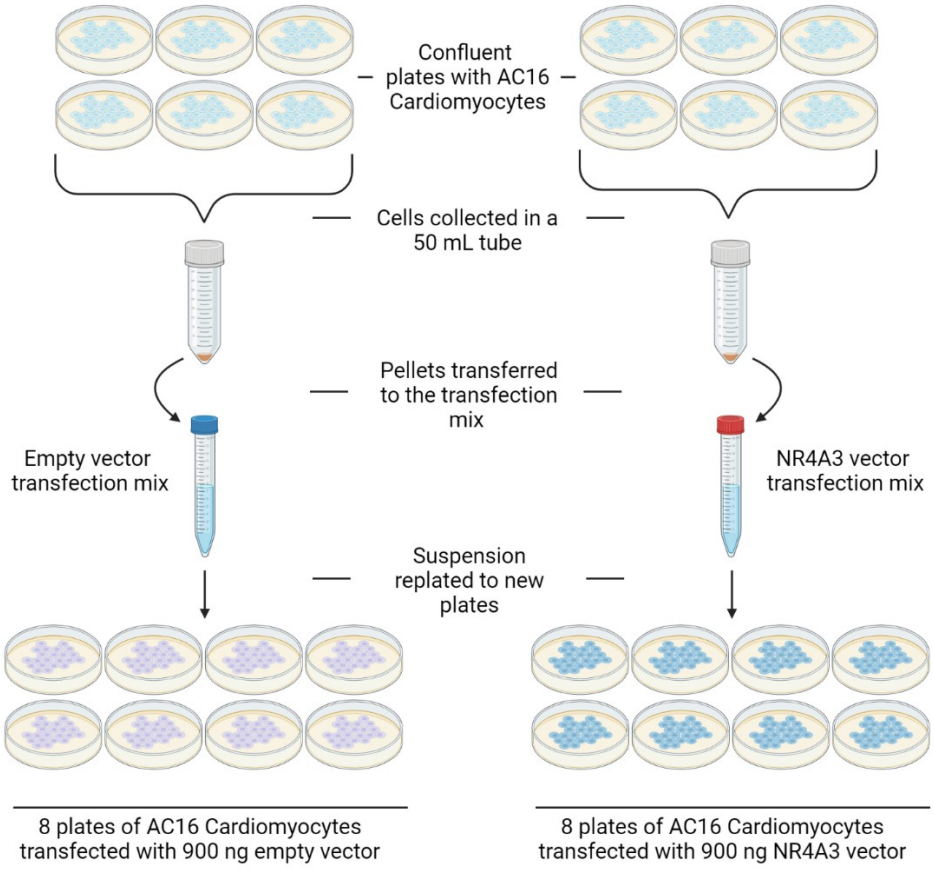

Figure 4: Transfection protocol. Confluent plates of AC16 cardiomyocytes (CMs) were collected and spun down to form a cell pellet. The pellets were transferred to either a transfection mix for the empty vector (EV) or NR4A3 vector. Following this, the cell suspension got replated to eight new plates for each vector. Created with BioRender.com.

3.1.2. Doxorubicin treatment

Doxorubicin hydrochloride (DOX; Sigma-Aldrich) was dissolved in sterile water to make a stock solution with a concentration of 1 mM. This was further diluted with culture medium, giving a final concentration of 5 µM. Four of the plates transfected with the NR4A3 expression vector, and four of the plates transfected with EV were treated with 10

mL culture medium containing 5 µM DOX. A nonstimulated vehicle control group was made by treating the rest of the plates with 10 mL culture medium containing sterile water instead of DOX. Figure 5 shows an overview of the treatment. All plates were then incubated for 12 hours at 37°C, and two empty plates containing culture medium with either DOX or sterile water were also incubated. These two plates were used during the LDH assay as blanks.

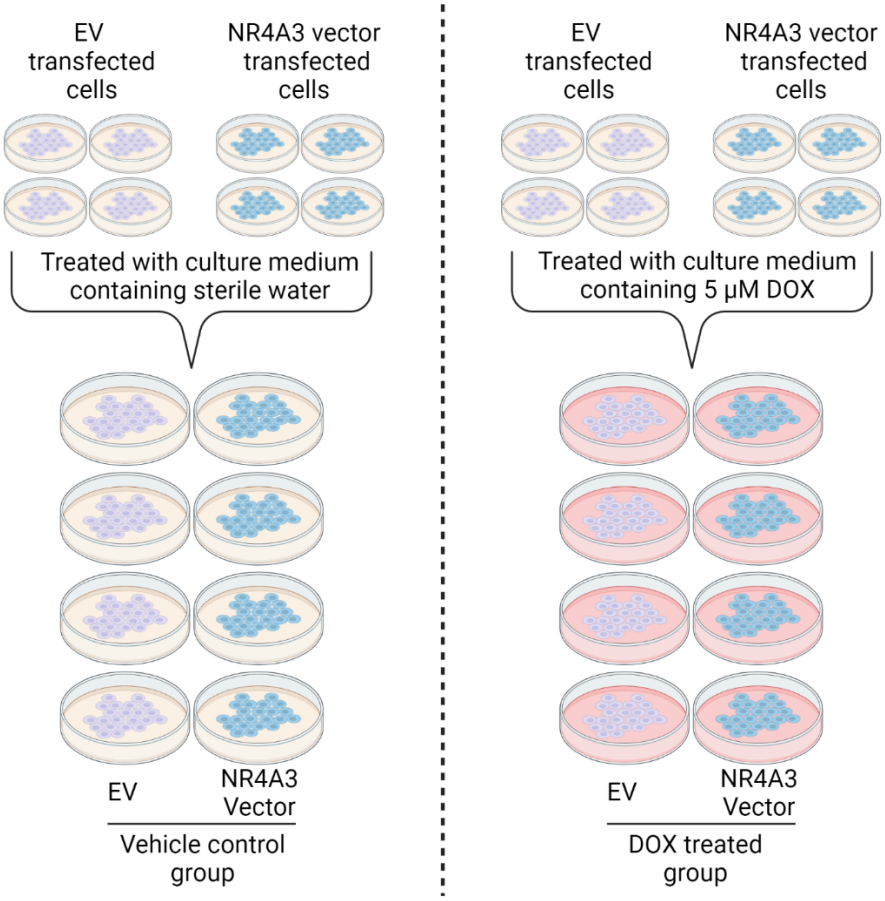

Figure 5: DOX treatment overview. Eight plates of AC16 cardiomyocytes (CMs), four transfected with empty vector (EV) and four transfected with NR4A3 expression vector, got treated for 12 hours with culture medium containing sterile water. This became the vehicle control group. Eight other plates got treated with culture medium containing 5 µM DOX for 12 hours. This was the DOX treated group. Created with BioRender.com.

The DOX concentration used in this study were based on a dose-response curve made earlier in this study. AC16 CMs were subjected to DOX-treatment for 12 hours with concentrations ranging from 0 to 5 µM. After 12 hours, an LDH assay was performed on the condition-medium. The results from the LDH assay showed that 5 µM of DOX resulted in a significant amount of LDH release (Fig. 6).

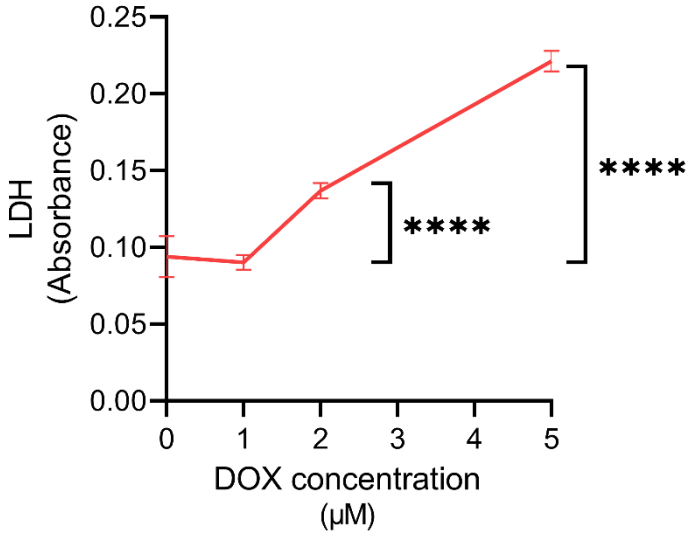

Figure 6: Dose-response curve of DOX on the amount of cell death in AC16 cardiomyocytes (CMs) determined by LDH assay. AC16 CMs were treated for 12 hours with different concentrations of doxorubicin (DOX), ranging from 0 to 5 µM. LDH release was determined by LDH assay after 12 hours, and absorbance was measured at 470 nm. Two biological replicates were used, with three technical replicates for each biological replicate. The graph is presented with mean ± SD. **** = $P \leq 0{,}0001$.

3.1.3. Cell harvest

After the 12 hours of incubation, the condition-medium from all 16 transfected plates with CMs was aspirated into separate tubes, later used during the LDH assay. The medium from the two empty plates containing culture medium with either DOX or sterile water was also collected. 1 mL of M-PER (Thermo Scientific) containing 1% Halt™ Protease Inhibitor Cocktail (Thermo Scientific) and 1% Halt™ Phosphatase Inhibitor Cocktail (Thermo Scientific) was added to each plate to lyse the cells. The plates were kept cold for at least 5 minutes, and then the cell lysate got collected into separate 1,5 mL tubes after scraping them off using cell scrapers. All the tubes containing cell lysate got centrifuged for 15 minutes at 12 000 g and 4°C. The supernatant then got transferred to a new set of 1,5 mL tubes, and these cell lysates were stored at -18°C.

3.2. Bradford assay

The Bradford assay is a colorimetric assay for the quantification of total protein concentration in a sample. By adding a Coomassie G-250 dye to a protein sample in an acid environment, the proteins will bind to the Coomassie dye, and the color changes from brown to blue based on the amount of protein. The absorbance can be measured at 595 nm, where a stronger blue color indicates more protein in the sample (95).

Bradford assay was performed by using the Pierce Coomassie Plus Bradford Assay Kit (Thermo Scientific). First, a standard was made by diluting 25 µL of Albumin Standard (2 mg/mL) in 1975 µL MilliQ H_2O. More standards were made through 1:2 dilutions of the first standard. 640 µL from each standard got mixed with 160 µL Pierce Coomassie Plus Assay Reagent and plated in triplicates in a 96-well plate. 640 µL of MilliQ H_2O were also mixed with 160 µL Pierce Coomassie Plus Assay Reagent and plated in triplicate. The dilution series now contained standards with known concentrations ranging from 0 µg to 4 µg of protein per milliliter.

All the 16 cell lysates from the cell harvest got prepared by mixing 10 µL of the cell lysate with 40 µL of M-PER. 4 µL of each dilution got mixed with 636 µL MilliQ H_2O, and a blank sample was made by mixing 4 µL M-PER with 636 µL MilliQ H_2O. All the dilutions and the blank got mixed with 160 µL Pierce Coomassie Plus Assay Reagent and plated in triplicates in the same 96-well plate as the standards. The absorbance was measured immediately with the FLUOstar Omega microplate reader at 595 nm, and a standard curve was made using the absorbance values from the standards. This was used to calculate the protein concentration in the 16 cell lysate samples.

3.3. Western blotting

Western blotting is a qualitative and semiquantitative method to detect specific proteins in a sample. By resolving the proteins on a gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins are separated from each other based on their molecular weight. A basic overview of the procedure is shown in figure 7. The gel is divided into the stacking gel and the resolving gel, where the stacking gel is more porous than the resolving gel and stacks the proteins so that the proteins loaded on to gel can enter the resolving gel at the same time. How much percentage of polyacrylamide in the gel is based on the molecular weight of the protein of interest. A common percentage is usually 10%, but very light proteins may need a higher percentage of polyacrylamide. After the electrophoresis, the proteins in the gel are transferred to a membrane, which is also known as blotting. This is done by laying a membrane on the gel and using an electric field oriented perpendicular to the surface of the membrane (96). There are different types of membranes to choose from, where nitrocellulose (NC) membranes are the most common. Another type of membrane is polyvinylidene fluoride (PVDF) that has several advantages over the NC membrane. The first is that PVDF membranes can be stripped and reprobed because it is not as fragile as the NC membranes. Another benefit is that there is no need for methanol in the transfer buffer when using PVDF membranes. Methanol is used in transfer buffers when using NC membranes to limit the precipitation of high molecular weight proteins. However, the PVDF membranes need to be pre-wetted in methanol before use because PVDF is highly hydrophobic (97).

Blocking is the next step after the proteins are transferred to the membrane. This is done by washing the membranes with 5% bovine serum albumin (BSA) or nonfat dried milk diluted in tris buffered saline-Tween 20 (TBS-T). Blocking prevents nonspecific binding of antibodies to the membrane (96). After blocking, a primary antibody is added to the membrane, specific for the protein of interest. A secondary antibody is then added, which is specific to the primary antibody. The secondary antibody is labeled, for example, with radioactive isotopes or enzymes (98). Horseradish peroxide (HRP) is a commonly used enzyme to conjugate to the secondary antibody. In the presence of hydrogen peroxide, HRP catalyzes the oxidation of luminol to a light-emitting product that can be detected using a camera. This is called chemiluminescence detection (99). The protein of interest will appear as a band on the membrane, varying in size based on the amount of protein present.

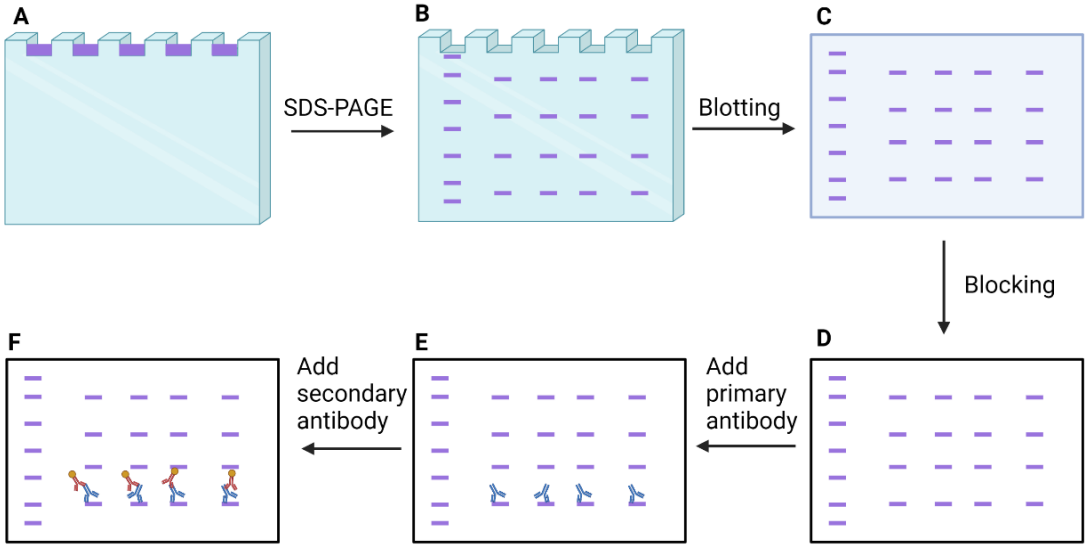

Figure 7: Basic overview of western blotting. A) Samples are loaded to the wells of a gel, where a protein marker is loaded to the first well. **B)** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate the proteins based on molecular weight. The bands on the gel are hypothetical proteins and are not visible. **C)** All the proteins in the gel are blotted to a membrane. **D)** To prevent nonspecific binding of the antibodies, the membrane is blocked with either 5% bovine serum albumin (BSA) or nonfat dried milk diluted in tris buffered saline-Tween 20 (TBS-T). **E)** A primary antibody specific for the protein of interest is added to the gel and binds to the protein on the membrane. **F)** To detect the primary antibody, a secondary antibody specific to the primary antibody is added to the membrane. The secondary antibody is conjugated with a dye or enzyme for detection. Created with BioRender.com.

In this study, twelve of the cell lysate samples from the cell harvest, three from each group randomly selected, got diluted to a protein concentration of 5 µg/µl using M-PER. The amount of M-PER added to each sample was based on the protein concentration from the Bradford assay. 200 µL of each sample then got mixed with 200 µL of 2x Laemmli Sample Buffer (Santa Cruz Biotechnology), giving the samples a final protein concentration of 2,5 µg/µL. These samples were stored at 4°C.

Western blotting was done by using western blotting equipment from Bio-Rad. The samples were loaded into the wells of a 15-well polyacrylamide gel, starting from the third well. The amount of sample loaded and the percentage of polyacrylamide in the gels varied based on the protein of interest (Table 1). MagicMark™ XP Western Protein Standard (Invitrogen) and Precision Plus Protein All Blue Prestained Protein Standard (Bio-Rad) were used as protein molecular weight markers. 5 µL of each was loaded to the second well. The loaded gels were placed in the electrophoresis chamber and filled with running buffer (25 mM Tris, 192 mM Glycine, 0.1 w/v SDS). Electrophoresis was run at 150 mA for 90 minutes for all the gels.

Table 1: Amount of protein loaded and gel percentage used during western blotting.

Protein of interest	*Amount protein loaded (µg)*	*Gel percentage (%)*
Beta-actin	30	10
Flag tag NR4A3	40	10
Phospho-Akt (Ser473)	40	10
Akt	20	10
Phospho-ERK1/2 (Thr202/Tyr204)	40	10
ERK1/2	20	10
Phospho-GSK-3β (Ser9)	50	10
GSK-3β	20	10
Phospho-STAT3 (Ser727)	60	10
STAT3	20	10
Bcl-xL	40	12
SOD2	50	12
Cyclin D1	30	10
cIAP2	100	10

After electrophoresis, the gels were blotted to PVDF membranes, which were pre-wetted in methanol. Blotting was done by stacking the gels together with PVDF membranes and placed in a transfer chamber filled with transfer buffer (25 mM Tris, 192 mM Glycine, pH 8.1 – 8.5). The transfer was run at 200 mA for 90 minutes for all proteins. Following the transfer, the membranes were washed three times with TBS-T for a total of 15 minutes before blocking with 5% nonfat dried milk in TBS-T for 60 minutes at room temperature. The membranes were then rewashed with TBS-T for 15 minutes before adding the primary antibody. This was done by diluting the primary antibody with 5 ml TBS-T for each membrane. Table 2 shows how much of each primary and secondary antibody used for each membrane. The membranes with the primary antibody were incubated overnight at 4°C on a shaker. After incubation, the primary antibody was collected, and the membranes were washed with TBS-T for 30 minutes. Based on which host the primary antibody came from, a matching secondary antibody conjugated with HRP was diluted in 10 mL TBS-T and added to the membrane. This was incubated for 90 minutes at room temperature on a shaker, followed by washing with TBS-T for 60 minutes. After this, the membranes were put in a 50:50 mix of SuperSignal West Pico PLUS Luminol/Enhancer Solution (Thermo Scientific) and SuperSignal West Pico PLUS Stable Peroxide Solution (Thermo Scientific) for one minute. The membranes were then imaged using the LI-COR Odyssey FC, and quantification was done with the Image Studio Software. Some of the membranes were stripped with 5 ml NaOH (0,5M) for 20 minutes to remove the antibodies from the membrane. After stripping, the membranes were blocked again and reprobed with new antibodies. Something to consider is that stripping is a harsh process and can remove some of the proteins on the membrane resulting in unreliable results (100).

Table 2: Dilutions used for the different primary and secondary antibodies.

Proteins of interest	*Host*	*Primary antibody dilution in 5 ml TBS-T*	*Secondary antibody dilution in 10 ml TBS-T*
Beta-Actin	Mouse	1:1000	1:3000
Flag tag NR4A3	Mouse	1:2000	1:3000
Phospho-Akt (Ser473)	Rabbit	1:1000	1:2000
Akt	Rabbit	1:1000	1:2000
Phospho-ERK1/2 (Thr202/Tyr204)	Rabbit	1:1000	1:2000
ERK1/2	Rabbit	1:1000	1:2000
Phospho-GSK-3β (Ser9)	Rabbit	1:1000	1:2000
GSK-3β	Rabbit	1:1000	1:2000
Phospho-STAT3 (Ser727)	Rabbit	1:1000	1:2000
STAT3	Mouse	1:1000	1:3000
Bcl-xL	Rabbit	1:2000	1:2000
SOD2	Rabbit	1:1000	1:2000
Cyclin D1	Rabbit	1:300	1:2000
cIAP2	Rabbit	1:500	1:2000

Since the transfected NR4A3 expression vector was tagged at the N-terminus with a 3xFLAG peptide, it was possible to verify if the transfection was successful by western blotting. The benefit of using antibodies specific to the flag tag instead of antibodies specific for the NOR-1 protein is that the antibody for the flag tag will only show the exogenous proteins. Western blotting confirmed that the cells had translated the NR4A3 expression vector to NOR-1 proteins using a primary antibody specific to the flag tag (Fig. 8).

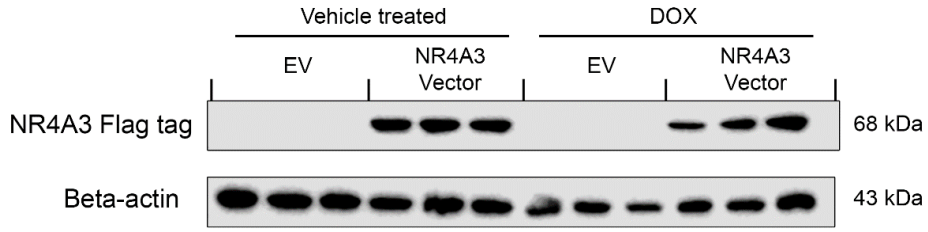

Figure 8: Verification of NR4A3 transfection. Western blotting confirmed that the NR4A3 expression vector had been successfully transfected to the AC16 CMs.

3.4. LDH assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme that catalyzes the conversion of lactate to pyruvate and back. The release of intracellular LDH into the culture medium is an indicator of cell membrane damage and irreversible cell death (101). By adding a tetrazolium salt (iodonitrotetrazolium violet) to the culture medium, the LDH converts this salt to a red formazan product. The amount of red color formed is therefore proportional to the amount of cell death (102). However, FBS has been shown to increase the LDH assay background signal and reduce the sensitivity for the assay (103). One solution is to use heat-inactivated FBS, but this could reduce the growth promotion capacity of FBS because the heat could inhibit growth factors. Instead, different batches of FBS were tested for LDH activity, and the batch with the lowest amount of LDH activity was used in the culture medium during DOX treatment before the LDH assay.

The CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega) was used to determine the LDH release from the cells. This assay was performed immediately after collecting the condition-medium during the cell harvest. 200 µL condition medium from each tube got diluted with 800 µL phosphate-buffered saline (PBS), giving it a 1:5 dilution. This dilution was done so that the absorbance levels did not exceed the plate-readers capabilities. 50 µL of the diluted condition medium was added to a well in a 96-well plate, and each sample had three technical replicates. Positive control was made by adding 1 mL of M-PER to a confluent plate with cells where the condition-medium was aspirated away just before adding the M-PER. The cell lysate got scraped from the plate, collected in a small tube, and kept on ice while being vortex mixed for five seconds every three minutes. After 15 minutes on ice, the cell lysate got diluted 1:100 with PBS and 50 µL of this dilution was added to three separate wells. A blank control for the positive control was made by diluting M-PER in a 1:100 ratio with PBS. This got added to three new wells. 50 µL of CytoTox 96® Reagent was added to each well, and the plate got foiled and incubated at room temperature for 60 minutes. 50 µL of stop solution was then added to each well, and the absorbance was immediately measured at 490nm using the FLUOstar Omega microplate reader.

3.5. MTT assay

The MTT assay is a cell viability assay used to indicate cell viability, proliferation, and cytotoxicity. The assay relies on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is a water-soluble yellow dye, to an insoluble violet formazan dye. This reduction is done primarily by the dehydrogenases and reducing agents present in metabolically active cells. Sodium dodecyl sulfate (SDS) is used to solubilize the formazan, where a darker purple color indicates a greater amount of viable, metabolically active cells (104). An alternative to the MTT assay is the ATP assay. The ATP assay is more sensitive than the MTT assay and does not require an incubation time after adding the assay reagents, reducing the number of times the viable cells go in and out of the incubator (105).

The MTT assay was done in a 96-well plate, so the AC16 CMs were transfected as previously described but seeded at a density of 30 000 cells per well. Two rows, 24 wells, were seeded with AC16 CMs containing the NR4A3 vector transfection mix, and two rows were seeded with AC16 CMs containing the EV transfection mix. Each well now contained 9 ng of the expression vector. The culture medium got changed after 6 hours and incubated for 24 hours. After this, the medium in half of the wells containing NR4A3 vector and EV got changed to culture medium containing 5 µM DOX. The other half received culture medium with sterile water instead of DOX. After 12 hours of incubation, the medium got aspirated away and replaced with 100 µL clear DMEM and 10 µL MTT Reagent (Roche). Three empty wells also received this to serve as negative blanks. Figure 9 shows an overview of the 96-well plate. Two hours later, 100 µL of Solubilization buffer (Roche) was added to each well to solubilize the formed formazan crystals. The absorbance levels in each well were determined at 595 nm using the FLUOstar Omega microplate reader the following morning.

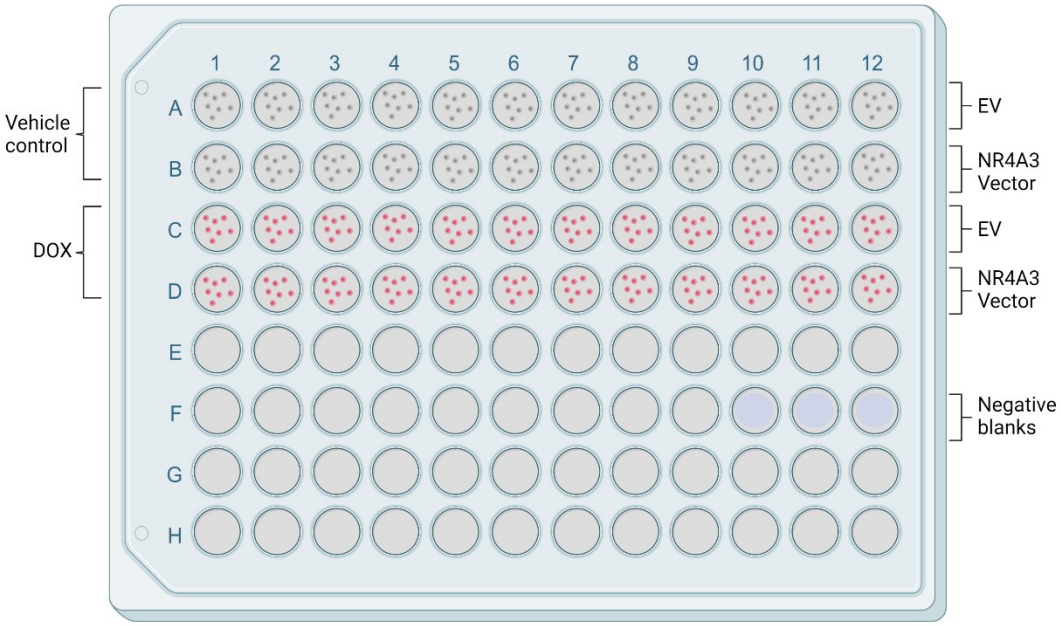

Figure 9: Overview of the 96-well plate used in MTT assay. Empty vector (EV) transfected AC16 cardiomyocytes (CM) were seeded in the wells of rows A and B. The wells in rows C and D were seeded with CMs transfected with the NR4A3 expression vector. CMs in rows A and B served as a vehicle control group, treated with culture medium containing sterile water, and CMs in rows C and D got treated with culture medium containing 5 µM of DOX for 12 hours. Created with BioRender.com.

3.6. Caspase-3 assay

Activation of caspase-3 is a critical factor for cell apoptosis. The amount of caspase-3 activity can be colorimetrically determined by adding a substrate for caspase-3 to the cell lysate (106).

The AC16 cells were transfected and treated with DOX the same way as previously described, but the cell harvest differed slightly as described below. After the DOX treatment, the condition-medium from all 16 plates got aspirated away, and 1 mL of trypsin was added to each plate. The cells got collected in separate 1,5 mL tubes and spun down at 500 g for 5 minutes. The trypsin then got aspirated away before the pellets in the tubes were resuspended in 250 µL lysis buffer (50 mM HEPES, 5 mM CHAPS, pH 7.4). This suspension got centrifuged at 12 000 g for 15 minutes at 4°C. The supernatant was aspirated to a new set of tubes, and these cell lysates were kept on ice while the protein concentration was determined with Bradford assay. Following the Bradford assay, 200 µL of each cell lysate got diluted to a concentration of 5 µg/µL, and 60 µL of each sample were added to a well in a 96-well plate in triplicates. To each well, 120 µL of assay buffer (20 mM HEPES, 1.62 mM CHAPS, 10 mM NaCl, 2 mM EDTA, pH 7.4) and 20 µL caspase-3 substrate (Merck) were added. Positive blanks were made by picking one sample from each of the four different treatment groups and mixing 60 µL of each with 100 µL of assay buffer, 20 µL caspase-3 substrate, and 20 µL caspase-3 inhibitor (Merck). A negative blank was made by mixing 60 µL of lysis buffer with 120 µL of assay buffer and 20 µL caspase-3 substrate. The absorbance levels in each well were determined at 405 nm using the FLUOstar Omega microplate reader the following morning.

3.7. Statistical analysis

Data are presented as means with SD. Testing for normality was done by using Shapiro–Wilk test. Differences between NR4A3 vector and empty vector in the control group and DOX group were analyzed using unpaired multiple t-tests corrected for multiple comparisons using the Holm-Sidak method. The statistical analyses were performed in GraphPad Prism (version 8.4.2). $P<0,05$ was considered significant.

4. Results

4.1. NOR-1 decrease cell death in DOX treated AC16 CMs

To determine if overexpression of NOR-1 could protect the cells against DOX-induced stress, we performed an LDH cytotoxicity assay. First of all, we confirmed that treatment of the AC16 CMs with 5 µM of DOX for 12 hours increased the LDH release within the EV-control treated groups (Fig. 10). In NR4A3 treated AC16 CMs, we found that DOX-induced LDH-release was significantly reduced compared to EV-treated cells (P<0,05). NOR-1 overexpression did not affect LDH release compared to EV control in unstimulated vehicle control conditions.

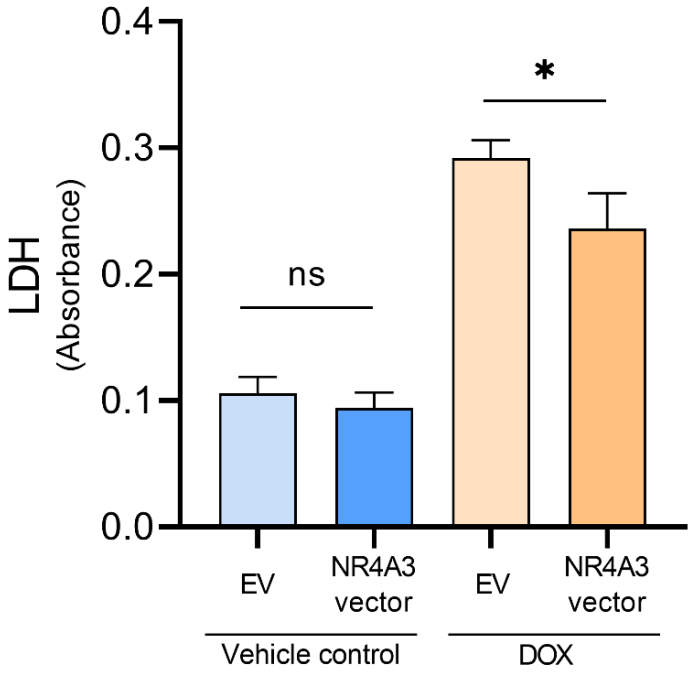

Figure 10: Effect of NR4A3 transfection on cell death measured by LDH release in DOX treated AC16 CMs. AC16 cells were transfected with expression vectors containing NR4A3 or an empty vector (EV) before treatment with 5µM of DOX for 12 hours. The amount of cell death was determined by measuring LDH release in the condition-medium after DOX treatment. Absorbance was measured at 470 nm. Four biological replicates were used in each of the four groups, with three technical replicates for each biological replicate. The graph is presented with mean ± SD. ns= P>0,05 (not significant), * = P≤0,05

4.2. NOR-1 increase cell viability in AC16 CMs

To determine if overexpression of NOR-1 would have any impact on cell viability, both in nonstimulated conditions as well as under DOX-induced stress, we performed an MTT assay. We found that DOX treatment led to decreased cell viability in AC16 CMs within the EV-treated groups (Fig. 11). Transfection of AC16 cells with the NR4A3 expression vector increased the cell viability in both DOX treated CMs (P<0,05) and in the unstimulated vehicle control CMs (P<0,0001). These results indicate that NOR-1 promotes the survival of DOX-treated AC16 CMs.

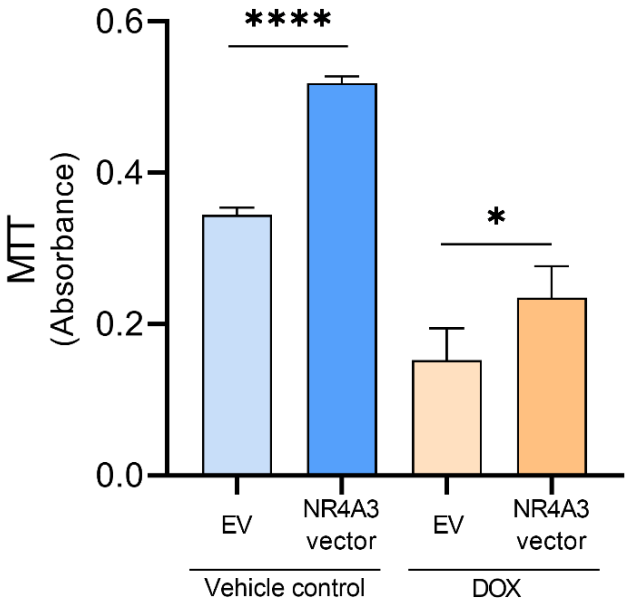

Figure 11: Effect of NR4A3 transfection on cell viability in DOX-treated AC16 CMs measured by MTT assay. Cells were seeded at 30 000 cells per well in a 96-well plate and transfected with 9 ng of vector per well. NOR-1 overexpression increased the cell viability in the CMs. Four biological replicates were used in the experiment, and each biological replicate was seeded in three different wells for each group, giving 12 wells for each treatment group. Absorbance was measured at 570 nm. The graph is presented with mean ± SD. * = $P \leq 0{,}05$, **** = $P \leq 0{,}0001$.

4.3. NOR-1 decrease DOX-induced apoptosis in AC16 CMs

Caspase-3 activation is one of the final mediators in the apoptotic pathway (107). Therefore, we wanted to determine if apoptosis is responsible for the DOX-induced cell death in AC16 CMs by performing a caspase-3 activity assay. We found that DOX treatment within the EV-control treated AC16 cells increased caspase-3 activity (Fig. 12A). Overexpression of NOR-1 induced a significant reduction in caspase-3 activity compared to EV-controls following DOX stress stimulation ($P<0{,}01$). In nonstimulated vehicle control conditions, overexpression of NOR-1 did not have any influence on the already low caspase-3 activity.

Cytochrome c release from the mitochondria is an upstream mediator of caspase-3 activation, and the anti-apoptotic protein Bcl-xL can block the cytochrome c release to cytosol (108). Therefore, we measured the protein expression levels of Bcl-xL (Fig. 12B). We found that DOX treatment decreased the expression of Bcl-xL, but overexpression of NOR-1 displayed a significantly higher expression of Bcl-xL than the EV-treated cells ($P<0{,}01$).

NOR-1 has shown to upregulate cIAP2, which is a potent inhibitor of apoptosis (71). We tried to measure the protein expression of cIAP2, but this was unfortunately not successful. Neither was the western blot for the pro-apoptotic protein Bax.

The results from the caspase-3 assay and the changes in protein expression levels of Bcl-xL indicate that apoptosis is a significant cause of cell death in DOX-treated AC16 cells and NOR-1 inhibits the apoptotic pathway.

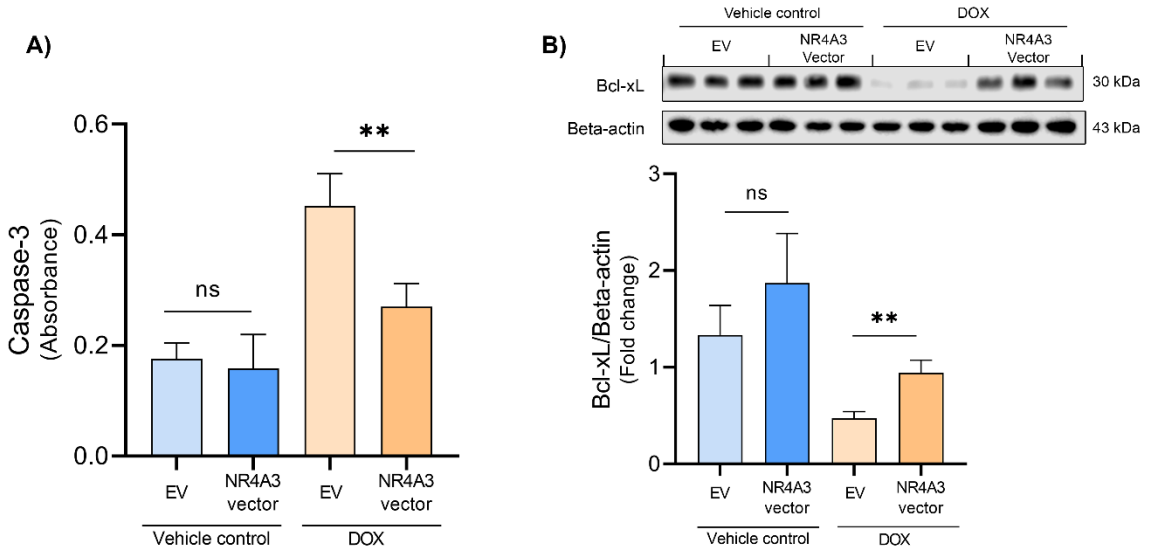

Figure 12: Effect of NR4A3 transfection on caspase-3 activity and Bcl-xL expression in DOX treated AC16 cells. A) AC16 cells were transfected with vectors containing NR4A3 or an empty vector before treatment with 5 µM of DOX for 12 hours. Caspase-3 activity assay was performed after treatment to determine the amount of apoptosis in each group. Absorbance was measured at 405 nm. Each treatment group consisted of four biological replicates and the caspase-3 activity assay was performed on three technical replicates from each biological replicate. **B)** Western blot showing NOR-1 attenuating the effect DOX treatment had on the expression of Bcl-xL (n=3). The graphs in A) and B) are presented with mean ± SD. ns= $P>0,05$ (not significant), ** = $P\leq0,01$

4.4. NOR-1 increase ERK phosphorylation in DOX treated AC16 CMs

The functional data show that NOR-1 protects the AC16 CMs against DOX-induced stress. To further determine the underlying signaling processes involved in the protective effect of NOR-1, we assessed the protein regulation of several key candidates previously reported to enhance resistance against cardiac stress. The first protein of interest was Akt, which is a part of the RISK pathway (109). Akt is a pro-survival kinase, and its phosphorylation at Ser473 is involved in the cardioprotective effect of ischemic preconditioning (110). Also, DOX has been shown to inhibit phosphorylation of Akt in a concentration- and time-dependent manner in H9C2 CMs (111). However, in the present study, we did not observe that DOX caused any effect on Ser473 phosphorylation of Akt in AC16 CMs (Fig. 13A). Furthermore, transfecting with NR4A3 did not seem to influence the amount of phosphorylation either.

Another prosurvival kinase in the RISK pathway is ERK (109). Earlier studies have shown that NOR-1 directly regulates the transcription of lipin 1, which is suggested to play a role in regulating cytosolic activation of ERK (69, 70). Also, activation of ERK has been shown to protect CMs against oxidative stress and DOX-induced apoptosis (112, 113). We found that treating the AC16 CMs with DOX resulted in decreased ERK phosphorylation on Thr202/Tyr204 (Fig. 13B). Interestingly, we found an almost three-fold increase in phosphorylation of ERK in the DOX-treated CMs transfected with NR4A3 versus the EV-treated CMs ($P<0.01$).

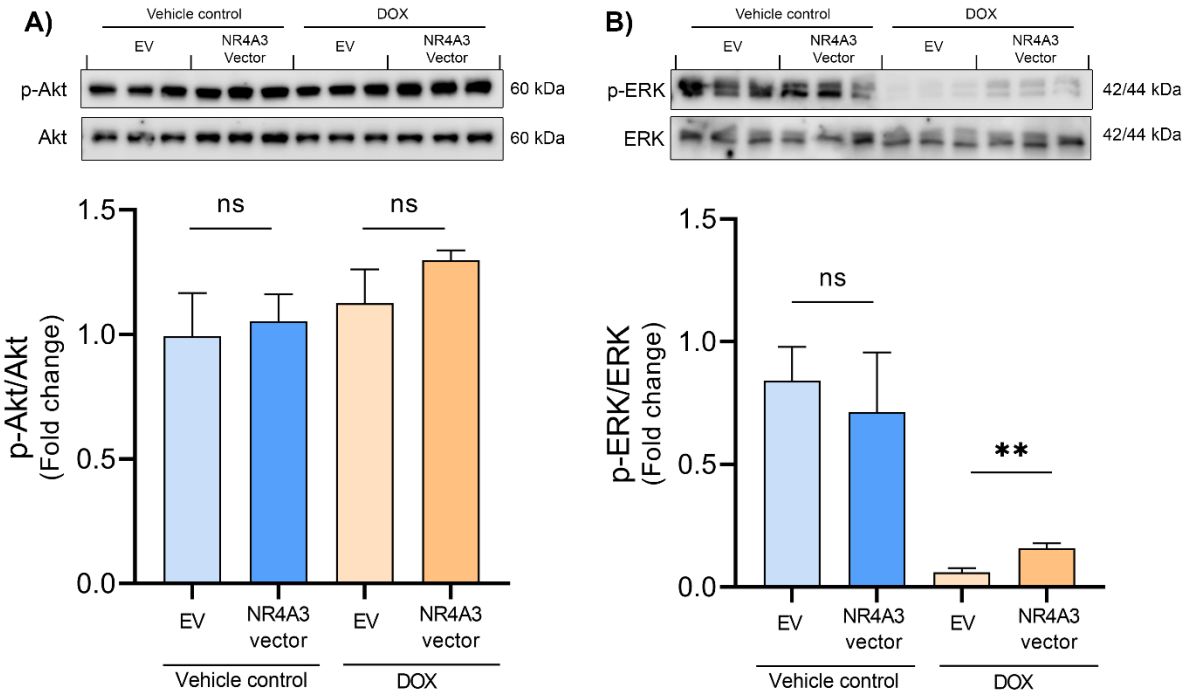

Figure 13: The amount of phosphorylation of Akt and ERK measured by western blotting
A) Treating the AC16 cells with DOX for 12 hours showed no effect on phosphorylation of Akt (Ser473) and neither did transfecting with NR4A3. **B)** DOX treatment showed a great decrease in phosphorylation of ERK (Thr202/Tyr204). The decrease in phosphorylation was significantly attenuated by transfecting with NR4A3 before DOX treatment. The graphs in A) and B) are presented with mean ± SD (n=3). ns= $P>0,05$ (not significant), ** = $P\leq0,01$

Furthermore, to evaluate the downstream targets of ERK, we assessed the phosphorylation of GSK-3β. ERK directly phosphorylates p90RSK, which in turn could inhibit GSK-3β through phosphorylation on Ser9 (114, 115). Our study found that NOR-1 overexpression showed a tendency to modulate the phosphorylation of GSK-3β on Ser9 in the DOX-treated CMs compared to the EV-control, but it was not significant ($P=0,068$, Fig. 14A). Furthermore, overexpression of NOR-1 did not significantly alter the phosphorylation of GSK-3β in our nonstimulated vehicle control group either.

Another protein of interest was STAT3. STAT3 plays a part in the SAFE pathway, and ERK is thought to be involved in the phosphorylation of STAT3 on Ser727 (116, 117). However, we did not observe any significant changes in phosphorylation of STAT3 on Ser727 in this study (Fig. 14B).

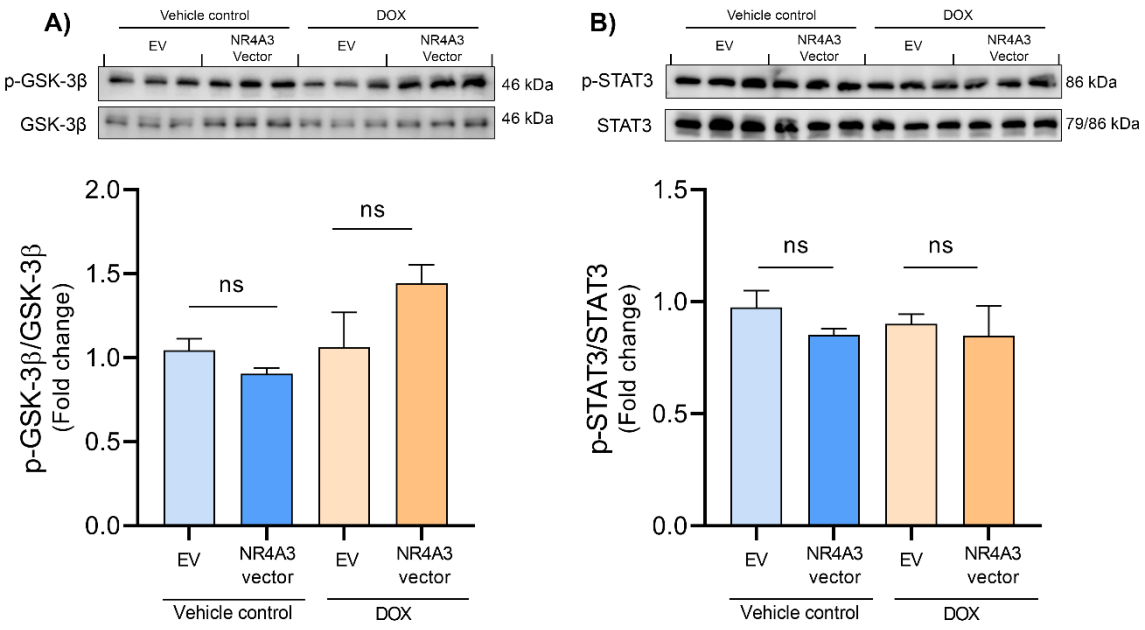

Figure 14: The amount of phosphorylation of GSK-3β and STAT3 measured by western blotting. The results in A) and B) shows that treating the AC16 cells with 5 μM DOX for 12 hours showed no effect on phosphorylation of GSK-3β (Ser9) and STAT3 (Ser727), and NOR-1 overexpression did not significantly alter the protein levels. The graphs in A) and B) are shown as mean with SD (n=3). ns=*P*>0,05 (not significant)

The increase in ROS production caused by DOX is known to be cardiotoxic, and SOD enzymes control ROS levels in the cells (118, 119). Therefore, we wanted to measure the protein expression of SOD2. However, we did not observe any significant changes in SOD2 expression after NR4A3 transfection (Fig. 15A).

We also explored the expression of cyclin D1. Inhibition of GSK-3β on Ser9 causes induction of cyclin D1, which plays a vital role in cell proliferation (120, 121). In our study, no significant changes in the expression of cyclin D1 between the different groups were observed (Fig. 15B).

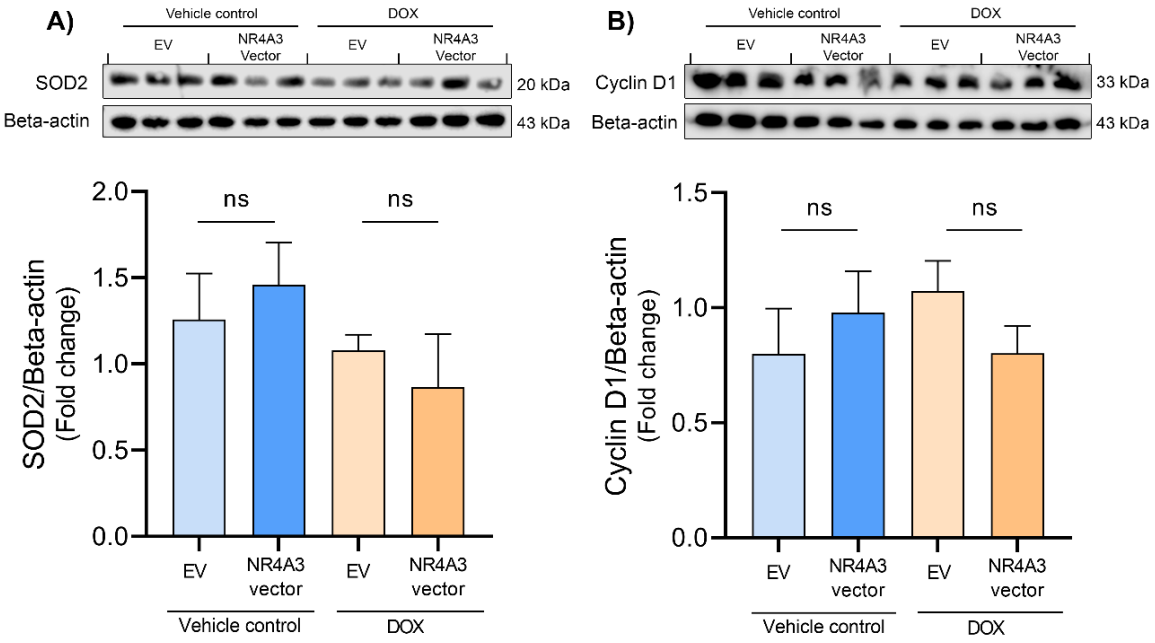

Figure 15: Protein expression of SOD2 and Cyclin D1 measured by western blotting. The results in A) and B) shows that treating the AC16 cells with 5 µM DOX for 12 hours showed no effect on protein expression of SOD2 and Cyclin D1, and neither did NOR-1 overexpression. The graphs in A) and B) are presented with mean ± SD (n=3). ns= *P*>0,05 (not significant)

5. Discussion

Further insight into mechanisms involved in cardioprotective measures against RI after an MI is essential to reduce mortality. The present study explored the effects of NOR-1 overexpression on cell death, cell viability, and apoptosis in AC16 CMs. Oxidative damage to the ischemic tissue caused by ROS has gained support as one of the primary mechanisms to explain the cellular damage caused by RI (14). Excessive ROS production has also been proposed as the primary mechanism in which DOX injure the myocardium (122). Therefore, we treated the AC16 CMs with 5 μM DOX for 12 hours to induce oxidative stress. In parallel, we explored the effects of NOR-1 overexpression in a nonstimulated vehicle control group. Following this, we investigated signaling pathways that might play a role in cardioprotection.

To our knowledge, there are no studies on NOR-1 in AC16 CMs to this day, making it difficult for us to compare our findings to earlier work. There are, however, several studies on DOX-treated AC16 CMs (123-125). Yuan et al. treated AC16 CMs with DOX and observed that cell death occurred in a time- and dose-dependent manner (123). In the initial parts of the present study, we evaluated the dose-response curve of DOX in AC16 CMs (Fig. 6). We found that treating the AC16 CMs with 5 μM of DOX for 12 hours increased the LDH release significantly. This treatment gave us a good model to explore the possible protective effects of NOR-1 overexpression.

Overexpression of NOR-1 decreased the amount of LDH release in DOX treated AC16 CMs (Fig. 10), indicating less cell death when overexpressing NOR-1. However, the increased LDH release in the culture medium only means that the plasma membrane is damaged and does not indicate if the cell death occurred by apoptosis, necrosis, or another form of cellular damage (126). In our nonstimulated vehicle control group, overexpression of NOR-1 did not influence the LDH release. The activity of NR4A orphan nuclear receptors are regulated by expression levels, protein-protein interactions, and posttranslational modifications (65). Our results indicate that the increased protein level of NOR-1 does not affect cell death in a nonstimulated condition but is involved in protecting the CMs during DOX-induced stress.

Caspase-3 activation is a strong indication of apoptotic cell death (106). The caspase-3 activity assay indicated that DOX-treated AC16 CMs underwent apoptotic cell death (Fig. 12A), which also was observed by Pan et al., who treated AC16 CMs with DOX (127). Furthermore, our caspase-3 activity assay showed that NOR-1 is a potent inhibitor of apoptosis in DOX-treated AC16 CMs. NOR-1 overexpression of the CMs resulted in significantly less caspase-3 activity compared to the EV-treated CMs. These data support the results from the LDH activity assay, indicating that NOR-1 plays a role in protecting the CMs through the apoptotic pathway during DOX-induced stress. Furthermore, NOR-1 overexpression in a nonstimulated condition did not alter the amount of caspase-3 activity significantly. This finding further strengthens our observations in the LDH assay that NOR-1 overexpression does not influence cell death during nonstimulated conditions. We did not explore if necrosis contributed to the total amount of cell death, but apoptosis and necrosis are both involved in DOX-induced cardiotoxicity (128). Further work needs to be done to explore if NOR-1 overexpression could protect the CMs against necrotic cell death.

We determined cell viability by MTT assay and observed decreased cell viability between the EV-treated CMs in the vehicle control and DOX treated groups (Fig. 11). Chen et al. also observed reduced cell viability when treating AC16 CMs with 5 μM DOX for 24 hours

(124). In our DOX-treated AC16 CMs, NOR-1 overexpression increased cell viability significantly, indicating that NOR-1 plays a positive role in cell viability during DOX-induced stress. Interestingly, NOR-1 overexpression also increased the cell viability in the nonstimulated vehicle control group. These findings could support our hypothesis that NOR-1 overexpression increases cell viability, which prepares the CMs for an eventual stress condition. In addition, NOR-1 overexpression did not alter apoptosis or cell death under nonstimulated conditions. NR4A3 is identified as one of the most exercise-responsive genes in skeletal muscle (63). Furthermore, exercise training reduces the damage in the heart during ischemia and reperfusion, but the molecular mechanism responsible for this cardioprotective effect remains unclear (129). Thus, the increased cell viability observed could serve as a link between the cardioprotective effects seen after exercise training and NOR-1, but further studies are needed to validate this theory.

Having observed that overexpression of NOR-1 protected the CMs against DOX-induced stress, the aim was further to explore the underlying signaling processes involved in the protective effect of NOR-1. First, we examined the protein expression of several pro-survival kinases involved in the RISK pathway known to protect the myocardium after IPC (109). According to several studies, Akt phosphorylation is an essential factor in cardioprotection during an MI after IPC (130, 131). Furthermore, DOX-treatment reduces the amount of Akt phosphorylation in rat hearts and the H9C2 cell line derived from rat heart tissue (132-134). The dephosphorylation of Akt after DOX-treatment is found to be mediated by protein phosphatase 1 (134). Surprisingly, DOX treatment did not dephosphorylate Akt in our AC16 CMs (Fig. 13A). One possible reason for this might be the duration of DOX treatment. In the study on the H9C2 cell line, the duration of DOX-treatment lasted for 24 hours, compared to 12 hours in the present study (132). Thus, a longer treatment time might be needed to modulate the phosphorylation of Akt in our AC16 CMs. However, our findings do not rule out the possibility for NOR-1 to phosphorylate Akt as a stress response.

Chen et al. found that both Akt and ERK phosphorylation protected CMs against DOX-induced apoptosis (113). In the present study, we observed that DOX dephosphorylated ERK and NOR-1 overexpression significantly attenuated this dephosphorylation (Fig. 13B). ERK is involved in the RISK pathway and exerts anti-apoptotic functions in CMs during stress (33). In addition, ERK inhibits apoptosis by preventing caspase-8 and caspase-9 activation in other cell types (135, 136) and could thus explain the observed effect of NOR-1 overexpression on caspase-3 activity in the present study.

Furthermore, ERK can also inactivate the pro-apoptotic Bad via p90RSK (137), which causes loss of BADs inhibitory effect over Bcl-2 and Bcl-xL (35). Our study observed that NOR-1 overexpression significantly increased the already low protein expression of Bcl-xL in AC16 CMs after DOX-treatment (Fig. 12B). This finding could explain the decrease in apoptosis seen in DOX-treated AC16 CMs by overexpression of NOR-1. Bcl-xL prevents cytochrome c release from the mitochondria resulting in inhibition of apoptosis. Another way Bcl-xL can inhibit apoptosis is by binding to cytochrome c in the cytosol and thereby prevent the formation of the apoptosome complex (108).

Furthermore, another target for ERK is GSK-3β. Erk can, also through p90RSK, phosphorylate GSK-3β and inhibit its function (36). The protective role of GSK-3β inhibition during myocardial ischemia and reperfusion is to increase the threshold for mPTP opening (138). In addition, the inactivation of GSK-3β might also prevent BAX from translocating to the mitochondrial membrane (139). Furthermore, GSK-3β inactivation in other cell types leads to an accumulation of β-catenin in the cytoplasm.

The accumulated β-catenin then translocates to the nucleus and modulates the transcription of genes such as cyclin D1 and c-myc (36). Hanh et al. overexpressed β-catenin in both CMs and cardiac fibroblasts and observed decreased apoptosis after an MI. The possible protective effects of β-catenin were through upregulation of Bcl-2, survivin, cyclin D1, and cyclin E2 (140). However, overexpression of NOR-1 did not seem to significantly modulate the protein levels of neither phosphorylated GSK-3β nor cyclin D1 in our DOX-treated AC16 CMs (Fig. 14A and Fig. 15B). The NOR-1 overexpression did show a tendency to phosphorylate GSK-3β during DOX-induced stress, but we can not draw any conclusion from this. Further studies are therefore needed to explore if NOR-1 overexpression alters GSK-3β phosphorylation.

ERK can also phosphorylate STAT3 at Ser727 (116), but we observed no significant changes in phosphorylated STAT3 levels in our study (Fig. 14B). Jiang et al. observed increased phosphorylation of STAT3 in H9C2 CMs after NOR-1 overexpression (66). However, this was the phosphorylation of STAT3 at Tyr705 and not Ser727. This Tyr705 phosphorylation of STAT3 resulted in a reduced inflammatory response after an MI. In further work, the phosphorylation of STAT3 at Tyr705 in AC16 CMs after NOR-1 overexpression should be explored. Furthermore, it might be STAT5 and not STAT3 that is responsible for the cardioprotective effects of the STAT family in humans (39).

The mechanism in which overexpression of NOR-1 could modulate the phosphorylation of ERK is unknown, but one theory is through the expression of lipin 1. NOR-1 directly increases lipin 1 expression in skeletal muscle (141). Furthermore, lipin 1 activates ERK in the cytosol, resulting in skeletal muscle regeneration (70). Lipin 1 is robustly expressed in the heart (69), but whether NOR-1 regulates lipin 1 expression in CMs needs to be determined in further work.

Exessive ROS-production has gained support as the primary cause in which DOX induces cardiotoxicity (142), and one of the scavengers of mitochondrial ROS is SOD2 (143). Chaiswing et al. demonstrated that SOD2 overexpression in mice protected against DOX-induced cardiotoxicity (144). Surprisingly, DOX-treatment did not alter the protein expression of SOD2 in our study (Fig. 15A). Cheung et al. showed that DOX treatment of H9C2 CMs resulted in a dose-dependent decrease in SOD2 expression (145). Similar to Akt, a longer treatment time might be needed to alter SOD2 expression. Another member of the SOD family, SOD1, has also been shown to decrease in expression levels after DOX treatment (146). Furthermore, exercise training increases the expression levels of SOD1 in cardiac tissue after exercise training and might play a part in protection against DOX-induced damage (147). One interesting finding by Alonso et al. performed in VSMC was that NOR-1 overexpression indirectly downregulated SOD2 but upregulated SOD1 (148). We did, however, not see any significant changes in SOD2 expression levels after NOR-1 overexpression (Fig. 15A), but we did not explore SOD1 expression levels. Our results could indicate that NOR-1 overexpression does not initially protect the CMs against DOX-induced stress through modulating SOD2 expression levels. Still, the influence of NOR-1 on other enzymatic antioxidants should not be ruled out.

We hypothesized that NOR-1 overexpression protected the CMs against oxidative stress. However, Alonso et al. showed that NOR-1 overexpression in VSMC modulated the expression levels of SOD enzymes, as mentioned, but they also showed that NOR-1 overexpression increased ROS production (148). In the present study, we did not measure ROS production, but it would be interesting to see if NOR-1 overexpression increased ROS production during nonstimulated conditions. Small amounts of ROS are known to act as a trigger for IPC (42), where a ROS-sensitive mechanism seems to be

involved in the cardioprotective effects of IPC in both rats and rabbits (149, 150). In further studies, a ROS detection assay should therefore be performed to investigate the ROS production in the different treatment groups.

One protein that triggered our interest as a possible factor for protecting the CMs against DOX-induced stress after NOR-1 overexpression was cIAP2. Alonso et al. reported that cIAP2 is a downstream effector of NOR-1 in VSMC exposed to hypoxic stress (71). cIAP2 is a member of the IAP family, with the ability to inhibit apoptosis (28). Zhang et al. showed in mice that cIAP2 suppressed the activation of caspase-8, which in turn decreased the activation of caspase-3 (151). Furthermore, Chua et al. reported that overexpression of cIAP2 in transgenic mice protected against myocardial RI (152). Unfortunately, we could not detect cIAP2 in our cell lysate samples during western blotting, even when loading the maximum amount of protein. There might have been a problem with the antibody used, but due to time shortage, we were not able to explore this further.

Even if our results indicate that NOR-1 overexpression protects CMs against DOX-induced stress, it does not directly mean that NOR-1 could protect the CMs against RI after an MI. DOX-induced damage shares similarities with RI, like increased ROS production, but DOX also inhibits Top2 (153). Top2α is undetectable in the heart (85). However, DOX also inhibits Top2β, which CMs do express (153). Therefore, another study on CMs exposed to RI, overexpressed with NOR-1, should be performed.

The main protective mechanisms of NOR-1 overexpression in DOX treated AC16 CMs, based on our findings, are proposed in figure 16. We observed increased ERK phosphorylation and increased protein levels of Bcl-xL. These findings could explain the decreased caspase-3 activity observed in this study. However, more work is needed to validate these results. We should not rule out the possibility of NOR-1 overexpression protecting the CMs through other signaling pathways not explored in this study.

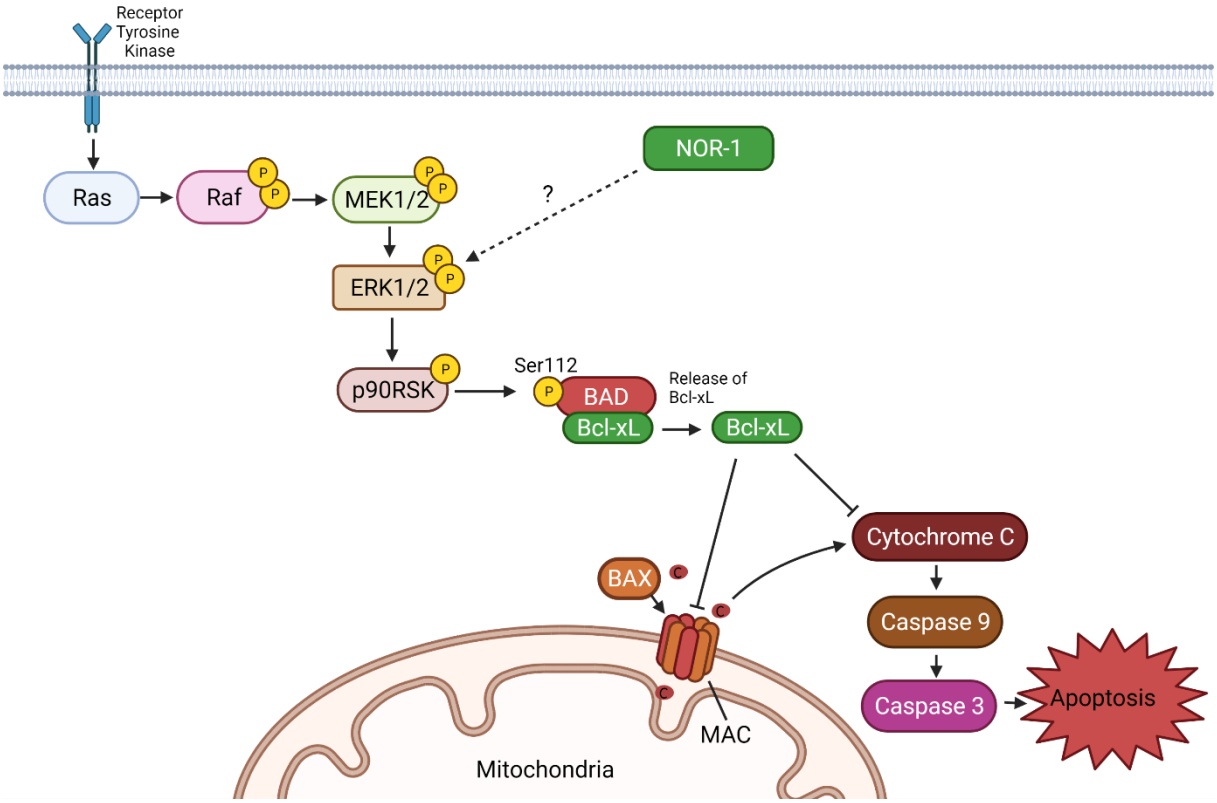

Figure 16: Proposed protective mechanism of NOR-1 overexpression in DOX-treated AC16 cardiomyocytes (CMs). Neuron-derived orphan receptor 1 (NOR-1) overexpression in DOX-treated AC16 CMs led to increased phosphorylation of extracellular signal-regulated kinase (ERK). The interaction between NOR-1 and ERK is currently unknown. ERK can, through p90 ribosomal S6 kinase (RSK), increase the amount of B cell lymphoma-extra large (Bcl-xL) (35, 137). Bcl-xl inhibits cytochrome c release from the mitochondria by preventing the formation of the mitochondrial apoptosis-induced channel (MAC) and inhibiting cytochrome c in the cytosol (108). In the end, this results in decreased caspase-3 activity and less apoptosis. Bax, Bcl-2-associated protein X; Bad, Bcl-2-associated death promoter; Ras, rat sarcoma; Raf, rapidly accelerated fibrosarcoma; MEK, MAPK/ERK kinase. Created with BioRender.com.

5.1. Limitations

A significant limitation in the present study is the lack of repeated experiments, resulting in low statistical power. Due to time limitations, we were not able to replicate the different experiments. More time would have given us the ability to optimize the methods and produced more experimental data, increasing the statistical power in this study. Several other proteins would have been interesting to explore, but issues with certain antibodies and time restrictions made this not feasible. Also, different kinds of assays could have given us further insight into the potential cardioprotective effects of NOR-1 overexpression. For example, flow cytometry could have been performed to explore the amount of necrosis in the different treatment groups (154). In addition, different concentrations and treatment times with DOX could also have provided valuable information.

Another limitation of this study was the use of a cell line and not primary cardiac cells. Cell lines do not entirely mimic primary cells, and experimental results can differ (90). However, primary cardiac cells are challenging to maintain in cell culture and require internal organ biopsy (155), compared to cell lines that are easily maintained and cost-effective (90). Therefore, our work should, in the future, be repeated in primary cardiac cells to validate our findings further.

6. Conclusion

The present study has given a unique insight into the potential protective effects of NOR-1 overexpression in CMs. We demonstrated that NOR-1 overexpression decreased cell death, apoptosis, and increased cell viability in DOX-treated AC16 CMs. This protective effect of NOR-1 overexpression may involve the phosphorylation of ERK and increased levels of Bcl-xL. Furthermore, we demonstrated that NOR-1 overexpression increased the cell viability of AC16 CMs during nonstimulated conditions without affecting cell death and apoptosis. Thus, our findings indicate that NOR-1 could serve as a potential cardioprotective protein in response to cellular stress.

There is still a lot to uncover about the functions of NOR-1 in CMs, both during normal and pathological states. Also, whether NOR-1 expression in the heart and the cardioprotective effects seen after exercise training are connected is still unknown. Further studies are therefore needed to answer these questions. However, our findings could serve as a basis for further work.

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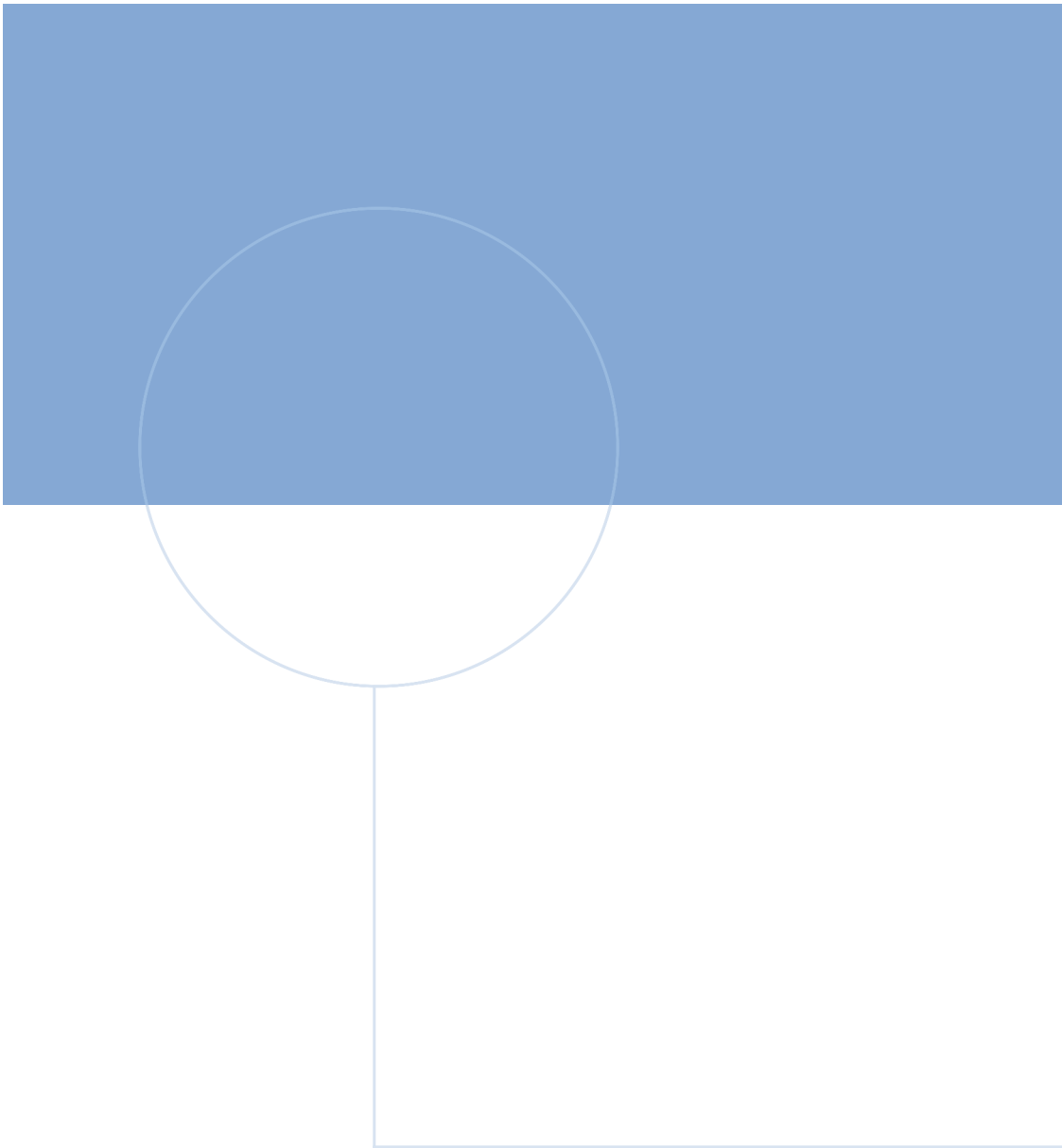
Appendix 1: List of reagents used

Reagent	Manufacturer	Catalog Number	Origin
AC16 cardiomyocytes	Millipore	SCC109	Darmstadt, Germany
Dulbecco's Modified Eagle's Medium	Gibco	11330-032	Grand Island, NY, USA
Fetal Bovine Serum	Gibco	10270-106	Brazil
Antibiotic Antimycotic Solution	Sigma	A5955	Israel
HyClone™ Trypsin	GE Healthcare	SH30042.02	South Logan, UT, USA
M-PER	Thermo Scientific	78501	Rockford, IL, USA
pReceiver-M12 expression-vector	GeneCopoeia	EX-Z0686-M12	Rockville, MD, USA
Control vector pReceiver-M12 expression-vector	GeneCopoeia	EX-EGFP-M12	Rockville, MD, USA
PolyFect Transfection Reagent	QIAGEN	301107	Hilden, Germany
Halt™ Phosphatase Inhibitor Cocktail	Thermo Scientific	78420	Rockford, IL, USA
Halt™ Protease Inhibitor Cocktail	Thermo Scientific	87786	Rockford, IL, USA
Doxorubicin hydrochloride	Sigma-Aldrich	44583	Saint Louis, MO, USA
Pierce Coomassie Plus Bradford Assay Kit	Thermo Scientific	23236	Rockford, IL, USA
Caspase-3 Substrate	Merck	235400	Darmstadt, Germany
Caspase-3 Inhibitor	Merck	235423-M	Darmstadt, Germany
2x Laemmli Sample Buffer	Santa Cruz Biotechnology	SC-286962	Dallas, TX, USA
MagicMark™ XP Western Protein Standard	Invitrogen	LC5602	Carlsbad, CA, USA
Precision Plus Protein All Blue Prestained Protein Standard	Bio-Rad	1610373	USA
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo Scientific	34580	Rockford, IL, USA
CytoTox 96® Non-Radioactive Cytotoxicity Assay kit	Promega	G1780	Madison, WI, USA
Cell Proliferation Kit I (MTT)	Roche	11465007001	Mannheim, Germany
DYKDDDDK Tag Monoclonal Antibody	Invitrogen	MA1-91878	USA
Anti-β-Actin Antibody	Santa Cruz Biotechnology	SC-47778	Dallas, TX, USA
Phospho-Akt (Ser473) Antibody	Cell Signaling Technology	9271	Danvers, MA, USA
Akt (pan) (C67E7) Rabbit mAb	Cell Signaling Technology	4691	Danvers, MA, USA
Phospho-GSK-3-beta (Ser9) (D3A4) Rabbit mAb	Cell Signaling Technology	9322	Danvers, MA, USA
GSK-3β (27C10) Rabbit mAb	Cell Signaling Technology	9315	Danvers, MA, USA

Reagent	Manufacturer	Catalog Number	Origin
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody	Cell Signaling Technology	9101	Danvers, MA, USA
p44/42 MAPK (Erk1/2) Antibody	Cell Signaling Technology	9102	Danvers, MA, USA
Phospho-Stat3 (Ser727) Antibody	Cell Signaling Technology	9134	Danvers, MA, USA
Stat3 (124H6) Mouse mAb	Cell Signaling Technology	9139	Danvers, MA, USA
Bcl-xL (54H6) Rabbit mAb	Cell Signaling Technology	2764	Danvers, MA, USA
Cyclin D1 Recombinant Rabbit Monoclonal Antibody	Invitrogen	MA5-14512	USA
cIAP2 Monoclonal Antibody	Invitrogen	MA5-14997	USA
SOD2 Recombinant Rabbit Monoclonal Antibody	Invitrogen	MA5-29578	USA
Goat anti Mouse IgG (H/L):HRP	Bio-Rad	STAR207P	USA
Goat anti Rabbit IgG (H/L):HRP	Bio-Rad	STAR124P	USA



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