HDAC inhibition Protects Cardiomyocytes by Modulating Expression of Opioid Receptor and Inducing Pro-survival Autophagy by Inhibiting Class I HDACs during Cardiac Ischemia/Reperfusion

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UNIVERSITA' DEGLI STUDI DI VERONA

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Abstract

Background and objectives:

The 20th century sees a revolution of translational medicine. By combining the genetics and bimolecular studies, many new drugs have been developed to treat infection, hypertension, heart failure and cancer. The use of percutaneous coronary intervention reduced the mortality and morbidity of acute coronary syndrome dramatically. However, there is no standard therapy available that can mitigate cardiac reperfusion injury, which contribute to around half of the infarct size. Prior studies showed that the activation of opioid receptors (OPRs), which are G protein-coupled receptors, induces cardioprotection both *in vitro* and *in vivo*. The exact mechanism of this protection is not clear yet. In addition, an FDA approved Histone deacetylase inhibitors (HDACi), SAHA, reduces infarct size significantly in a rabbit ischemia/reperfusion (I/R) injury through autophagy when it is given at the time of the reperfusion. We will test whether opiate receptors protect myocardium through activating autophagy and whether HDAC inhibition regulates opiate receptor expressions.

Hypothesis:

We hypothesize that HDAC inhibition Protects Cardiomyocytes by Modulating Expression of Opioid Receptor and Inducing Pro-survival Autophagy by Inhibiting Class I HDACs during Cardiac Ischemia/Reperfusion and opiate receptors is downregulated in diabetic heart.

Methods:

Immortalized human ventricular myocytes (AC16) and human ES cell derived cardiomyocytes (hES-CMs) were treated with either DMSO or SAHA (2μM) 16 hours before subjecting to simulated I/R. Plate was imposed by a buffer exchange to ischemia-mimetic solution and were placed in a humidified gas chamber equilibrated with 95% N2, 5% CO2 to simulate ischemia. After 2 hour ischemia, reperfusion was initiated by buffer exchange to normoxic culture media with DMSO or SAHA and incubates in 95% room air, 5%CO2 for various times. The expression of delta or kappa opiate receptor (DOP and KOP) protein and mRNA was evaluated by Western Blotting and mRNA real-time quantitative PCR (qRT-PCR), respectively before and after I/R. In addition, nine C57BL6 wild-type mice were randomized into 3 groups: DMSO control, SAHA pretreatment (one day prior and at surgery), and SAHA treatment at the time of reperfusion only after surgery. Each surgery group was subjected to I/R surgery for 45min coronary ligation and 24h reperfusion. Another set of thirteen C57BL6 wild-type mice were

randomized into two groups, DMSO and SAHA pretreatment group without IR. To generate mouse type II diabetic model, we subjected mice to 50mg/Kg streptozotocin IP X 3 days then to high fat diet for 6 weeks. The blood glucose levels have been verified elevated compared with chow diet mice without STZ. Heart tissues from these mice were extracted and expression level of OPRs proteins and mRNA were determined by WB and qRT-PCR.

Results:

1. In AC16 cells, simulated ischemia reduces DOP and KOP expression dramatically around 80 %, and their levels recover almost completely in 2 hours after reperfusion (N=3, P≤0.005). Block of autophagy does not affect I/R regulated opiate receptor expression. Autophagy is downregulated after ischemia around 80% and partially recovers during reperfusion around 70 % (N=3, P≤0.005).
2. In AC16 cells, SAHA increases DOP and KOP expression around 50% and maintains the autophagy flux two folds during I/R by Western blots (N=3, P≤0.005). In mouse heart, SAHA pretreatment for 24 hours increases DOP and KOP protein around 85% and mRNA expression 3.5 folds by Western blots and qRT-PCR(N=3, P≤0.005). In mouse heart, SAHA reperfusion only treatment for 24 hours increases DOP and KOP expression around 65% by Western blots (N=3, P≤0.005).
3. The expression level of DOP and KOP protein and mRNA are downregulated around 50% in the diabetic mouse heart by Western blots and qRT-PCR (N=3, P≤0.005) respectively. The autophagy level is down-regulated around 63% in diabetic mouse heart (N=3, P≤0.005).

4. In AC16 cells, Class I and II HDAC inhibitor, SAHA and Class I HDAC inhibitor, Apicidin induce two-folds autophagic flux (N=3, P≤0.005). While class II HDAC inhibitor, MC1568 does not induce autophagic flux. We observed similar results in hES-CMs.

Conclusion:

Delta and Kappa opiate receptors expression levels are actively regulated by ischemia reperfusion. HDAC inhibition increases DOP and KOP expression. In diabetic heart, DOP and KOP expression are reduced. Class I HDAC inhibition induces autophagic flux in cardiomyocytes.

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List of Abbreviations and Synonyms

OPRs: Opioid Receptors

GPCR: G Protein-Coupled Receptors

FDA: Food and Drug Administration

HDACi: Histone Deacetylase Inhibitors

HAT: Histone Acetyltransferases

IS: Infarct Size

I/R: Ischemia/Reperfusion

AC16: Immortalized human ventricular myocytes

hES-CMs: Human Embryonic Stem Cell Derived Cardiomyocytes

DMSO: Dimethyl Sulfoxide

SAHA: SuberaniloHydroxamic Acid

TSA: Trichostatin A

DOP: Delta Opioid Receptor - Oprd1 **(δ)**

KOP: Kappa Opioid Receptor- Oprk1 **(κ)**

MOP: Mu Opioid Receptor **(μ)**

NOP: Nociceptin Receptor

QRT-PCR:Quantitative Real-TimePCR

STZ: Streptozotocin

IP: Intraperitoneal Injection

WHO: World Health Organization

IHD: Ischemic Heart Disease

CVDs: Cardiovascular Diseases

Sec: Second(s)

IPC: Ischemic Preconditioning

KATP: Potassium Channels

Akt : Protein Kinase B

PKC: Protein Kinase C

PI3K: Phosphoinositide 3-Kinase

GIK: Glucose-Insulin-Potassium

MPP: Mitochondrial Pores

IGF II: Insulin Growth Factor

IP3: Inositol Triphosphate

DAG: Diacylglycerol

EGFR: Epidermal Growth Factor Receptor

DMP: Diabetes Mellitus Patient

CABG: Coronary Artery Bypass

CHD: Coronary Heart Disease

CPB: Cardiopulmonary Bypass

BFA: Baflomcycin

LDH: Lactate Dehydrogenase

Chapter I

Introduction and Background

Cardiovascular medicine grows exponentially in the 20th century, ranging from coronary intervention, coronary bypass surgery, heart transplantation, statin medication and effective heart failure medical treatment. However, despite such progress, cardiovascular disease continues to be the number one cause of death in the world and is estimated to be around 17 million deaths every year. The high cost of cardiovascular disease increasingly affects state budgets throughout of the world.

Today we are able to better understand the heart structure and function, their mutual relationship and integration with the whole organism thanks to the integration of basic research with clinical medicine, known as Translational Medicine. It combines the genetics and bimolecular cellular studies yielding potential results that can be translated into the industry for the development of new drugs capable of protecting the myocardium especially in the cases of acute ischemia.

The heart contains highly heterogeneous and well organized cells, among them 2-3 billion heart muscle cells, however, they account for less than a third of the total number of cells in the heart. The rest are represented by a wide range of additional cells, including smooth muscles, endothelial cells, fibroblasts, and more recently, pluripotent cardiovascular "stem cells" which are all considered as permanent constituent, in contrast the impermanent cells which include lymphocytes and macrophages [1]. These distinct cells are not isolated from each other but indeed contribute to structural, electrical, biochemical, and mechanical properties of a functional heart and interact physically through soluble paracrine, autocrine and endocrine factors (Figure 1) [2].

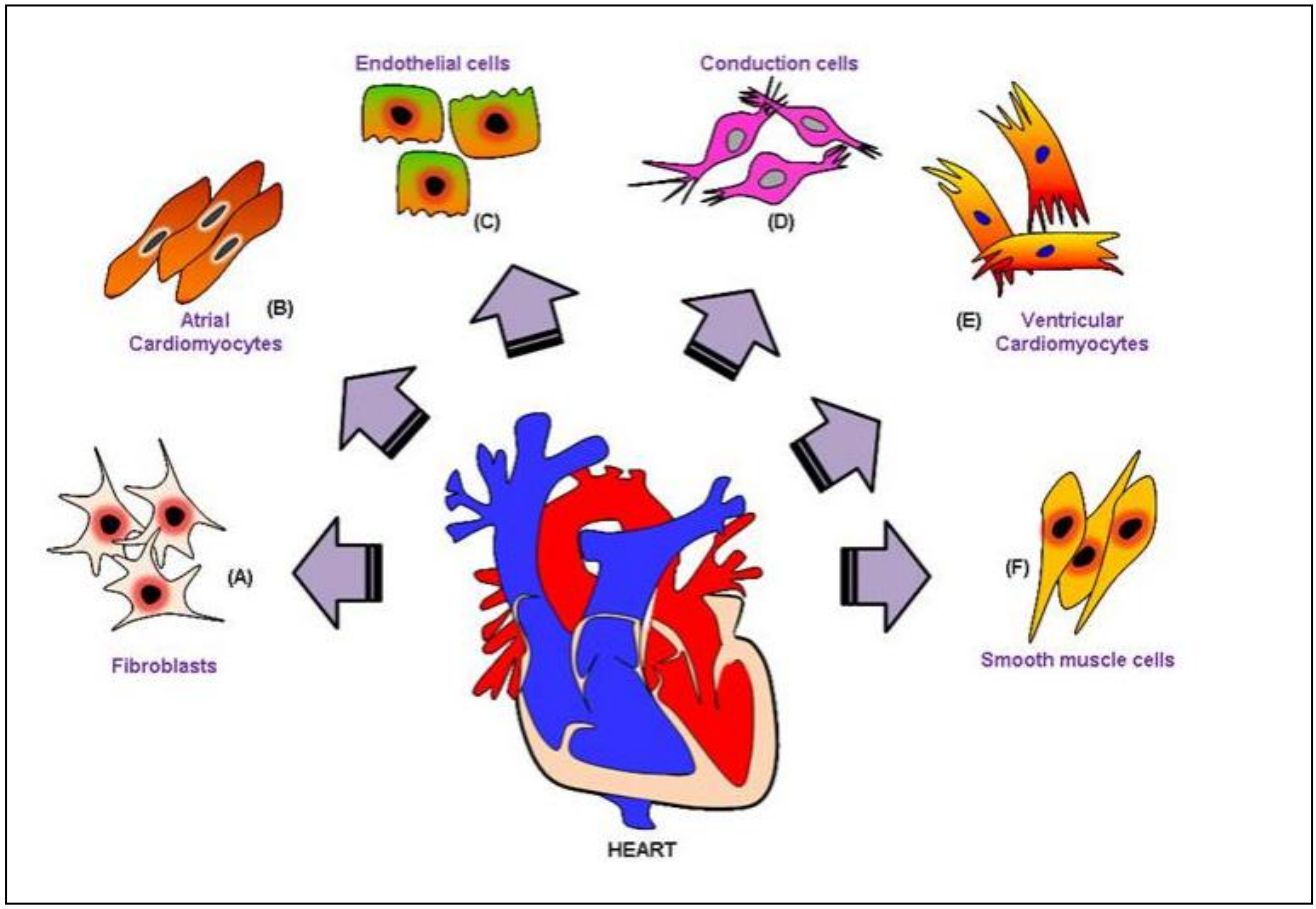


Figure 1. Schematic presentation of cardiac cell types. The adult heart consists of several cell types, which maintain structural, mechanical, electrical and functional integrity of the heart. **A.** Fibroblasts contribute in the formation of extracellular matrix, which provides mechanical support to the heart. **B.** Atrial cardiomyocytes contribute to contractility of atrium. **C.** Endothelial cells form the inner lining of cardiac blood vessels. **D.** Conduction cells generate electrical impulses for cardiac contractility. **E.** Ventricular cardiomyocytes are involved in contractility of the ventricles. F. Smooth muscle cells render support to the coronary arteries and vasculatures [3].

Ischemic Heart Disease (IHD)

Cardiovascular disease represents the leading cause of death, morbidity and mortality and responsible for 17% of all health care related costs (Figure.2) [4, 5]. Ischemic heart disease (IHD), which comprises of primarily coronary heart disease, is the prime manifestation of cardiovascular diseases (CVDs) and causes 46% of mortality in men and 38% in women [6]. It is a leading cause of death worldwide and has become a true epidemic that respects no border [4]. In 2012, out of 17.5 million people died from CVDs, 7.4 million people died of IHD (Figure.2) [7]. Three-fourths of global deaths due to coronary heart disease occurred in the low and middle-income countries [8]. The burden is expected to increase more in these countries in 2030[7, 8].

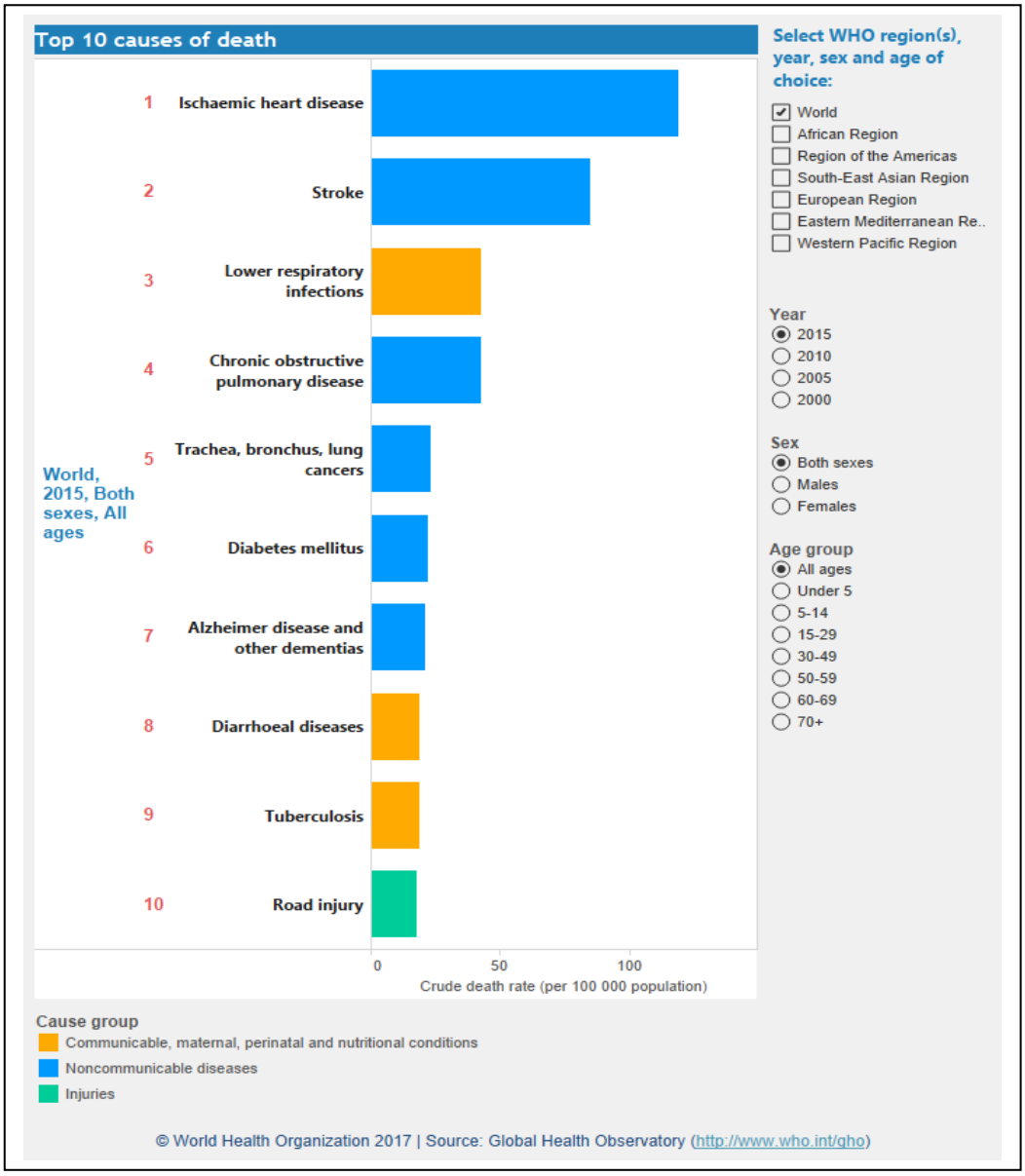


Figure 2. Ischemic heart diseases: The first cause of the death in the world [9].

Ischemia/Reperfusion Injury

Cardiac ischemia is a condition in which heart muscle (myocardium)receive inadequate blood flow, so causing a shortage of oxygen and glucose needed for cellular metabolism and inadequate removal of metabolic wastes .It is generally caused by problems with blood vessels, with existing damage or dysfunction of tissue. It could be asymptomatic or may cause chest pain, known as pectoris. This is most frequently results from atherosclerosis, which is the long-term accumulation of cholesterol-rich plaques in the arteries. many risk factors contribute to CVDs, such as smoking, aging, high cholesterol, high blood pressure, diabetes, obesity, and family history with CVDs [10].All of which lead to many path physiological conditions,

including myocardial infarction, peripheral vascular insufficiency, stroke, and shock [8].

Myocardial Cells response rapidly to ischemia stress, depending on the duration of ischemia, cell damage can be reversible or irreversible resulting in cell death. The Irreversible ones include mitochondrial collapses, energy depletion, large increases in intracellular calcium and extracellular potassium due to ion-pump deficiency, and cell swelling which all end up to a cell death[11]. During the ischemia phase, the myocytes undergo a number of modifications, such as reduction of high energy phosphates , glycogen depletion, lactate buildup, acidosis and mild intracellular edema (Figure 3.) [12].When re-establishing arterial flow (within 15 minutes), this phase of cellular suffering can be reversed by recapturing the damaged myocytes to their function. This reperfusion period, on the one hand, restores aerobic metabolism and promotes the rescue of myocyte, on the other it exacerbates (reversible) damage, developed during the ischemia period that can lead to new damage that causes post myocardial dysfunction [13, 14]. If reperfusion does not occur within the period of reversible ischemia and persists over time, subsequent metabolic alterations contribute to the transition from reversible damage to irreversible damage that manifests as necrosis and apoptosis [15].

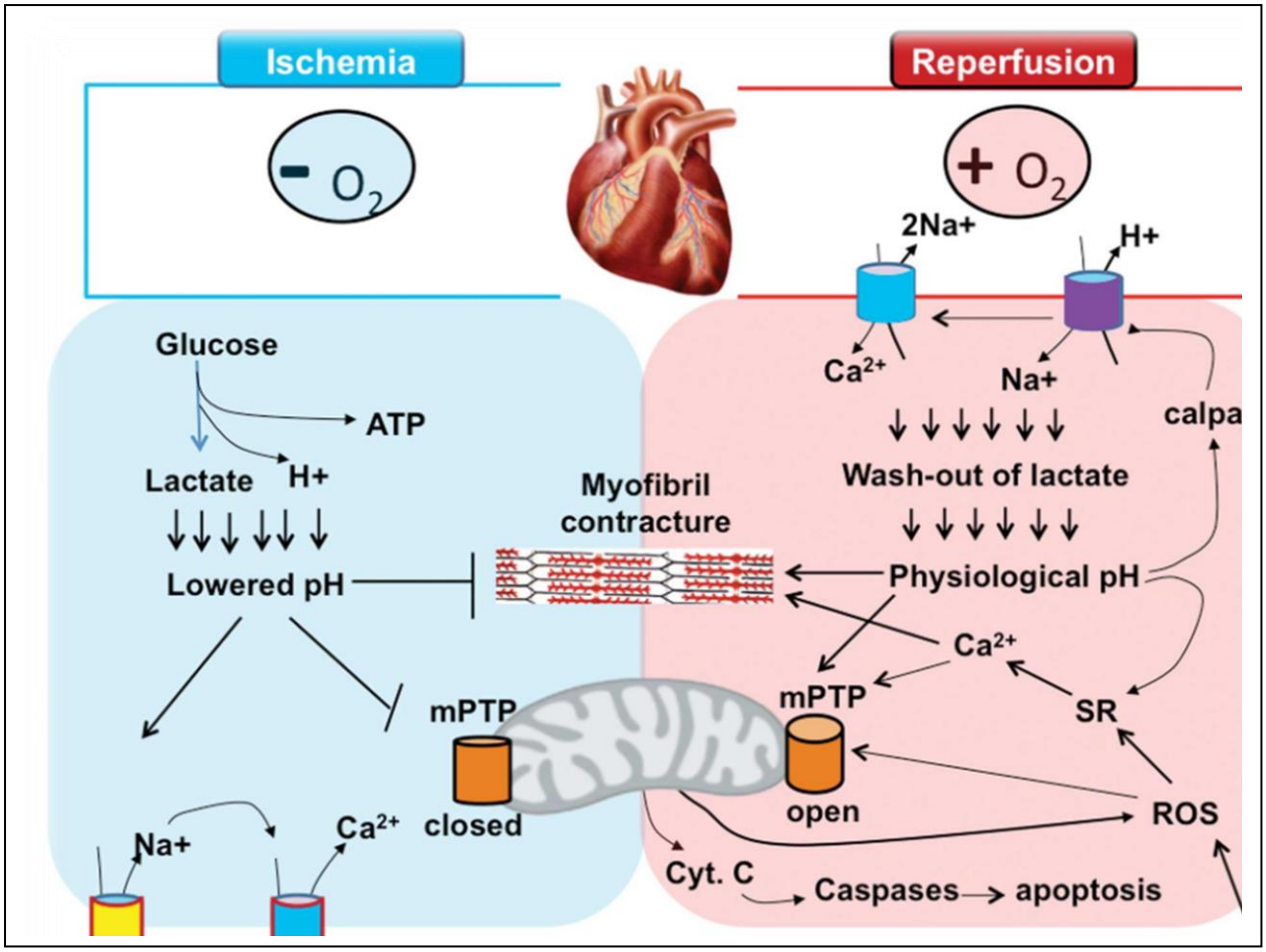


Figure 3. Ischemia/Reperfusion Phases: The myocytes undergo a number of metabolic modifications. mPTP, mitochondrial permeability transition pore; SR, sarcoplasmic reticulum; Cyt cytochrome c [16].

Acute Ischemia

The complete occlusion of a coronary artery, if prolonged inevitably leads to an infraction [17]. At the level of local metabolism, ATP synthesis is entrusted to exogenous sources such as fatty acids. With the cessation of blood flow, tissue oxygen is greatly reduced and only small amounts of oxygen are still present in erythrocytes trapped in capillaries and bound to myoglobin. Oxygen release is rapidly consumed in the mitochondrial electron transport chain by "closing" the oxidative respiration cycle in mitochondria. It has been shown that cessation of mitochondrial respiration occurs already after 2 sec from the onset of global ischemia in the isolated rat heart [18, 19]. Ischemia induces energy metabolism to move from aerobic to anaerobic metabolism. Concurrently with the inhibition of mitochondrial oxidative metabolism, ischemia causes immediate reduction in ATP, glucose-6-phosphate, and increases the availability of AMP and inorganic phosphate. In turn, these metabolites and modulators

increase the activity of phosphorylase and phosphofructokinase causing acceleration of glycogenosis and anaerobic glycolysis with concomitant lactate and proton production [20]. An important feature of ischemia is therefore the rapid decline in ATP [21-23]. During the ischemia period, anaerobic glycogenosis's becomes the first process to regenerate ATP in the ischemic cell. At this stage, the contractile force of the ischemic cells also decreases considerably when passing from aerobic to anaerobic metabolism [24-26].

I/R injury preconditioning

An important observation seen in an acute myocardial ischemic model is that short periods of ischemia did not lead to cell death as have been seen in Acute and prolonged ischemia[27-29]. This phenomenon has been called ischemic preconditioning (IPC) and becomes one of the most effective myocardial protection strategies against episode of ischemia [30]. Today, ischemic preconditioning has become the paradigm of cardioprotection on which some pharmacological strategies are currently based. It has been suggested that ischemic insults increase calcium levels in the cytosol during early stages of post-ischemic and reperfusion due to sodium-calcium pump depletion [27-30]. However, the mechanism of action of ischemic preconditioning remains not fully understood.

Adenosine was the first element identified in the protective mechanism by acting both as a trigger and as a mediator of ischemic precondition. This effects was abolished when a non-selective antagonist were used [28].Different types of receptors are well known to be act by adenosine that has a wide range of effects that make it more complex to explain the mechanism protection. The central role of adenosine appears to be through cellular coupling of the receptors for A1 and / or A3 and PKC by activating ATP-sensitive potassium channels (KATP).In addition to adenosine, there are other potential receptor-dependent and independent triggers as possible pathway signal pathways that may be responsible for induced cardioprotection [31]. Among this receptors are opioids, an Intracellular interaction of DOP and adenosine A1 receptors is indicated as an example of transactivation of GPCRs [32]. It is also thought that both delta and kappa OPRs which act via cellular mechanisms involving activation of ATP-sensitive (sarcolemma) k+ channel via G(1/o) proteins, phosphatidylinositol pathway via activation of kinase C, and most likely cross talk between adrenergic and OPRs in cardiomyocytes [31]. Nowadays, a number of studies have shown that the involvement of protein kinase C (PKC) in mediating anti- necrotic and anti-apoptotic actions of OPRs agonists [33, 34]. Studies of Maslov and colleagues have demonstrated that PI3 and Akt kinases are

involved in the cardioprotective effect of opioids [32, 34].Several studies have implicated bradykinin as a candidate trigger for preconditioning as preconditioned hearts show an increase of the interstitial bradykinin level associated with an increase of nitric oxide production and opening of KATP channels [35-37].

Also, it has been shown that activation of the δ-opioid receptor by opioid peptides is as a potent exogenous trigger of preconditioning in isolated human cardiac tissue [31] .Many reports have suggested that KATP channels are the downstream mechanism of cardioprotection, but the relative contribution of either sarcolemma or mitochondrial KATP channels to the triggering phase remains unclear. It has also been shown that by selectively activating the δ-opioid receptor with DPDPE, a selective agonist, the survival time in mice increased under hypoxic environments [38, 39]. Gross et al also demonstrated that ischemic PC is mediated by activation of the δ-opioid receptor, and by employing BNTX, a receptor antagonist, the infarct-limiting effect of IPC was abolished [40, 41].

Pharmacological conditioning

Many drugs were tested in experimental and clinical studies as novel approaches against ischemia –reperfusion mainly during cardiac surgery. Desfluorane is a volatile anesthetic used in patients undergoing elective coronary artery that have been shown to reduce A1C myocardial specific enzymes and to better preserve the postoperative contractility[42]. Also other volatile anesthetics such as sevofluoroin and isofluoroin have shown protective effects in animal experiments and reduce the level of myocardial apoptosis [43].

Adenosine has also been shown to have cardioprotective effects in patients undergoing myocardial revascularization, but the latter has not yet reached a consolidated clinical use. Moreover, its anti-apoptotic effect remains unclear [44].Other experimental and clinical evidence of cardioprotection occurred with the infusion of glucose / insulin / potassium (GIK) solutions in patients undergoing aortic valve replacement and myocardial revascularization. The GIK solution seems to prevent apoptosis at least in an experiment that induced an ischemia / reperfusion in an animal model[45].Interesting is the use in a cardiac surgery of a well-known drug as the main immunosuppressant in organ transplants such as cyclosporine A[46].This drug acts as a specific inhibitor of mitochondrial pores (MPP) and therefore acts as an anti-apoptotic drug through the action of bradykinin. Antioxidants and calcium antagonists and anti-inflammatory drugs such as corticosteroids have all shown protective effects in clinical trials [47].In experimental models, intracoronary infusion of insulin-like growth factor (IGF II)

appears to prevent apoptosis and the release of myocardial specific enzymes such as troponin I. Minocycline, a tetracycline family antibiotic, and some peptides have been shown to have anti-apoptotic effects in experimental models [48, 49].

Opiate receptor in I/R injury preconditioning

Manglik and colleagues have described opiates as most efficient analgesic drugs for long decade. In order to be activated, They bind to opioid receptor (OPRs) [50].Opioid receptors are G-protein coupled receptors(GPCRs),that regulate neurotransmission and can activated by both endogenously produced opioid peptides or exogenously administered opiate compounds [51]. This receptors include four major subtypes,i.e.mu(MOP),delta(DOP),kappa(KOP),and nociceptin receptor(NOP)identified by molecular cloning. Their proposed names based on the first ligand that was found to bind to the receptors [52].Each of the cloned OPRs is derived from a single gene; however, alternative spliced variants from their own genes have been isolated [53]. The three classic closely related subtypes, mu, delta and kappa, share similar sequence identity in their helices structures [54, 55] (Figure.4, Table. 1), with more variations in extracellular loops and very little similarity in their amino and carboxyl terminal[51, 56].Anatomical and molecular studies indicate their presence in many peripheral tissues such as, heart[57, 58],intestines, adrenal medulla, kidney, lung, spleen, testis, ovary and uterus [59], skin[60] .

Major families of the opioid system are derivatives of the endogenous peptides pre-proopiomelanocortin, pre-proenkephalin A and pre-proenkephalin B [61]. Furthermore, opioids, sometimes considered as neurotransmitters, and possess autocrine, paracrine, or endocrine functions in the peripheral tissues [33, 62, 63].

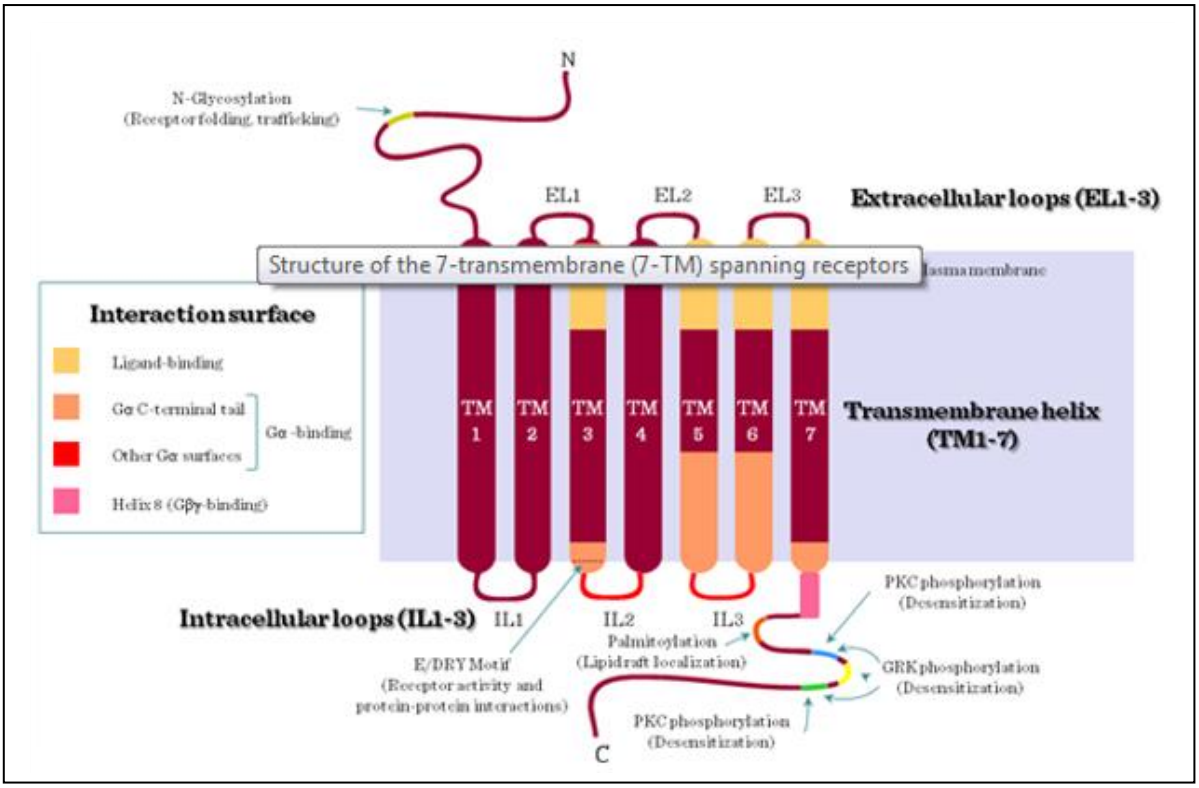


Figure 4. **Opioid receptors helices structure**. Seven transmembrane domains, the extracellular and intracellular loops that is important for receptor activity and protein-protein interactions[64].

Table 1. Opioid receptor types and classification [65].

Classification	Receptors			
	MOP	DOP	KOP	NOP
	μ	δ	κ	
	OP_3	OP_1	OP_2	OP_4
Subtypes	μ_1, μ_2	δ_1, δ_2	κ_1, κ_2, κ_3	
Prototype agonist	morphine	Ala-leu-enkephalin	ketocyclazocine	
Endogenous ligand	Leu-enkephalin	Leu-enkephalin	dynorphin	nociceptin
	Met-enkephalin	Met-enkephalin	β-endorphin	orphanin FQ peptide
	β-endorphin	β-endorphin		

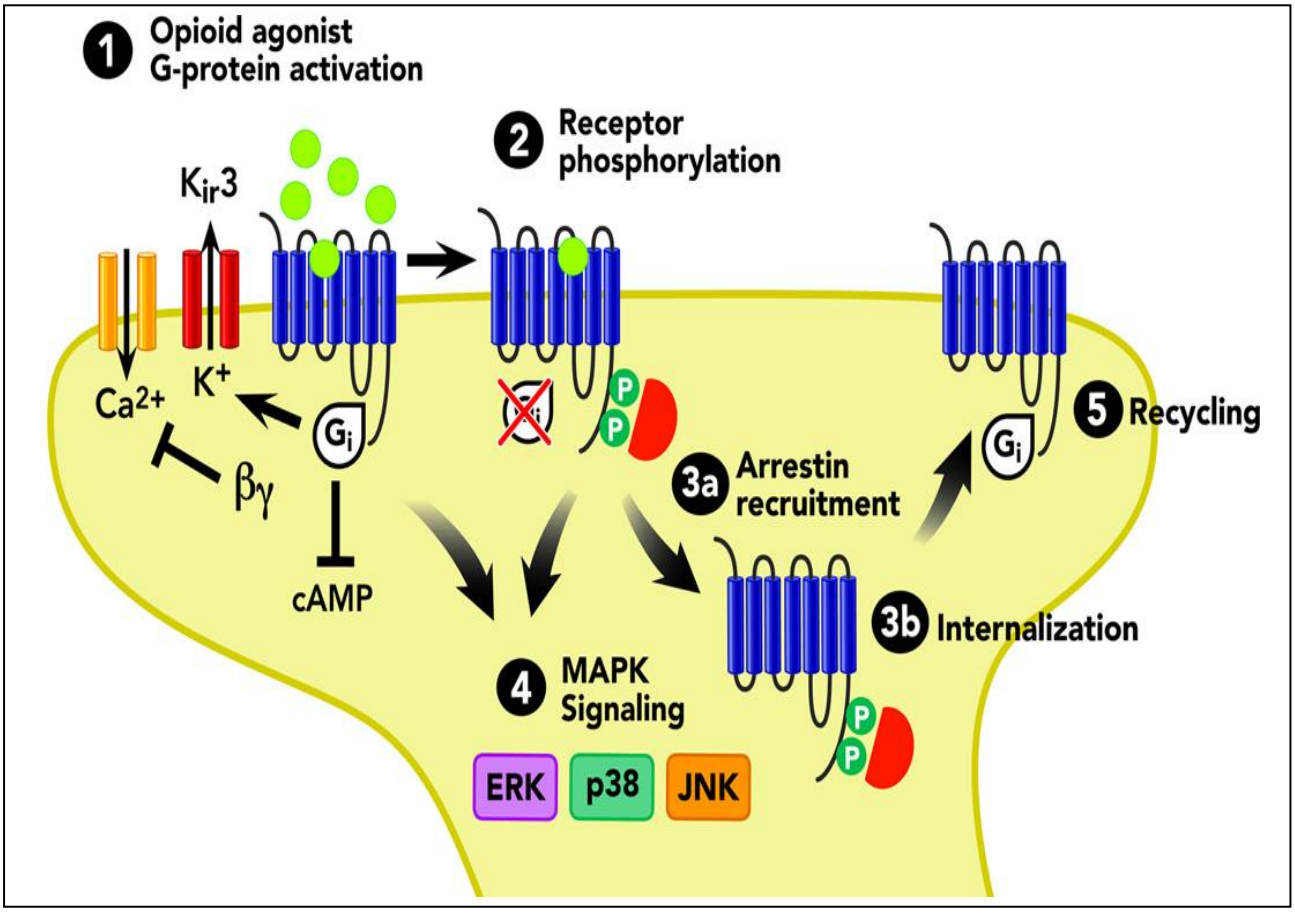
Myocardial opioid expression

As a high level of endogenous opioids is expressed in the heart [66, 67], many researchers were aimed to better understand their link with cardiovascular disease conditions such as myocardial infarction, stunning, and arrhythmia. They have been shown to regulate cardiovascular function in the healthy and diseased heart [68]. Clinically opioid are given to patients with advanced heart failure [69] as painkiller and their effects on cardiovascular function are most known to reduce arterial hypotension and bradycardia[70]. The term ischemic preconditioning and cardioprotective effects by OPRs was mentioned in many publications in different animal models [31, 71].

Opioid peptides synthesis and releases by myocardial were variable in different cell condition, influenced by aging and disease state[72] preserved and activated by I/R [73], and many are related to a greater generation of endogenous DOP selective ligands [66, 67] have shown that ventricular myocardium contain highest levels of pre-proenkephalin and that may explained the heart as an important neuroendocrine organ. The term ischemic preconditioning and cardioprotective effects by OPRs were mentioned in many publications in different animal models [71].

On a cell surface a series of chemical and physical events reactions produce biological responses once an opioid binds to their receptors, including proliferation, cell differentiation, metabolism alteration, cell growth, division, survival, and apoptosis [74]. Intracellular signaling molecules diffuse to their spatial target molecules in the cytosol and/or nucleus resulting in programmed changes in gene expression [75]. They have three major classes such as cyclic nucleotides (e.g. cAMP&cDMP), Inositol triphosphate (IP3), Diacylglycerol (DAG), and calcium ions (Figure 5).

Figure 5. Binding of opioid and activation of G-proteins. Opioid receptor triggers



activation of G-protein, which then activates a specific second messenger [76].

Functions of Opioid Receptors

Opioids are well known in pain modulation and widely associated with analgesia for postoperative pain therapy [77].In the heart, it has been shown an elevation of β-endorphin following muscle injury and hemorrhagic shock in naïve rats models [78].

The delta and kappa opioid receptors have been shown to mediate cardioprotection by preconditioning with myocardial ischemia and metabolic inhibition [54, 71].Evidence regarding the role of local opioids and opioid receptors in regulation of cardiovascular physiology and I/R has shown that activation of opioid receptors in the reduction of myocardial I/R by selective δ-opioid agonists when given acutely before ischemia and reperfusion [79].In a study, using administration of a potential and selective kappa opioid agonist, it has also shown antiarrhythmic effects depending on the activation of the κ-opioid receptor [80].

Opioid receptors, especially, DOP, mediate neuroprotection against ischemic injury. Even though there have been major controversies in the past decade on the role of opioids in the neuronal responses to ischemic insults by activation and inhibition of opioid receptors, recent data have clarified their neuroprotective effects against ischemic neuronal injury [81, 82]. The up-regulation of DOP expression and activation increase the neuronal tolerance to ischemic stress through triggering different mechanisms (PKC-ERK-Bcl2), and stabilization of ionic homeostasis [81] that reduce oxidative [83] and glutamate-induced [84] injury to reserve neuronal survival [83]. DOP also play a crucial role in neurogenesis. It is indicated that DOP agonist (SNC80) promotes neural differentiation from multipotent neural stem cells [85].

Opioids sometimes, act like cytokines to modulate the immune response in central and peripheral neurohumoral systems [86]. OPRs stimulation exerts suppression in numerous parts of the immune defense responses[87]. Opioid modulation of the immune response is mediated via the direct interaction with OPRs expressed by immune cells [88].They are also involved in regulation of ionic homeostasis under normoxic and ischemic conditions by intracellular elevation of Ca2+ or inhibition of their entry.

Opioid receptors are also involved in regulation of feeding in animal. Stimulation of OPRs increases feeding, while inhibition of OPRs reduces food intake in rodent models of obesity [89].

It has been well established that opioids trigger respiratory depression in humans and animals by a direct action on respiratory generating and high densities of OPRs brain areas [90, 91]. The use of opioid drugs for pain relief results in a respiratory depression that creates a significant clinical problem for patients treated with the drugs in the postoperative period [90].The massive release of endogenous opioids or overdose of opioid drugs can cause a severe respiratory depression and may be lethal. On the other hand, excessive use and abuse of opioid compounds lead to opioid tolerance/addiction in the nervous system via desensitization and internalization which greatly affects body homeostasis and brain physiology[92].

Opioid receptors cardioprotective action signal mechanism

Mechanisms of OPRs which are involved in cardioprotection are not clearly understood. It was supported that the occurrence of convergent pathways in which multiple GPCRs interact independently and transactivate epidermal growth factor (EGF) receptor-dependent kinase signaling to provide cytoprotection [34]. Intracellular interaction of DOP and adenosine A1 receptors is indicated as an example of transactivation of GPCRs [32].It is also thought that both delta and kappa OPRs which act via cellular mechanisms involving activation of ATP-sensitive (sarcolemma) k+ channel via G(1/o) proteins, phosphatidylinositol pathway via activation of kinase C, and most likely cross talk between adrenergic and OPRs in cardiomyocytes [33]. It is known that Gi/o proteins are intermediary linkages that provide cellular signaling between OPRs and protein kinase C (PKC).

Nowadays, a number of studies have shown that the involvement of protein kinase C (PKC) in mediating anti- necrotic and anti-apoptotic actions of OPRs agonists that involve PI3 and Akt kinases are mediating the cardioprotective effect of opioids [32, 34]. Besides, important roles of MEK1/2, ERK1/2, Src and JAK2 kinases and transactivation of OPRs in the cardioprotective effect of opioids in the development tolerance of the heart to ischemia and reperfusion are indicated and opioid transactivation of epidermal growth factor receptor (EGFR) is a connecting link between OPRs and ERK1/2 and PI3 kinase cascades [34].The activation of the EGFR increases the Akt (protein kinase B) and Pl3 (phosphatidylinositol-3-kinase) in their activities [93](Table 2).

Table 2. Summary of opioid receptor-dependent signaling [76].

Receptor	Cascade/Signaling Pathway	Model	Reference
μ Opioid	↑ ERK 1 and 2 (GRK-3 and arrestin dependent)	*In vivo* (murine)	Macey *et al.* 2006[67]
	↑ ERK 1 and 2 (arrestin dependent)	Astrocytes	Miyatake *et al.* 2009[68]
	↓ ERK 1 and 2 (chronic activation)	Astrocytes	Ikeda *et al.* 2010[69]
	↑ JNK 2 (PKC dependent)	*In vivo* and HEK293	Melief *et al.* 2010[35] Tan *et al.* 2009[70]
	↑ Stat3 phosphorylation	*In vivo* (murine) and CMT-93	Goldsmith *et al.* 2011[71]
κ Opioid	↑ ERK1 and 2	Astrocytes	Belcheva *et al.* 2005[72]
		In vivo	Bruchas *et al.* 2006[48] McLennan *et al.* 2008[73] Bruchas *et al.* 2008[74] Potter *et al.* 2011[75]
	↑ p38 MAPK (dependent on GRK-3 and arrestin)	Striatal neurons	Bruchas *et al.* 2006[48]
		Astrocytes	Bruchas *et al.* 2007[76]
		In vivo	Xu *et al.* 2007[77] Bruchas *et al.* 2011[78]
	↑ JNK 1	*In vivo*	Melief *et al.* 2010[35] Melief *et al.* 2011[32]
	↑ JAK2/STAT3 and IRF2 signaling cascade	PBMC	Finley *et al.* 2011[79]
δ Opioid	↑ ERK 1 and 2	HEK293	Eisinger *et al.* 2009[80] Eisinger *et al.* 2004[81] Audet *et al.* 2005[82]
	↑ ERK 1 and 2 (integrin stimulated, EGFR mediated)	HEK293	Eisinger *et al.* 2008[83]
	↑ ERK 1 and 2 (integrin stimulated, Trk1 mediated)	NG108-15	Eisinger *et al.* 2008[83]
	↑ PI3K/AKT/ ↓ GSK-3β	DOR-transfected CHO cells	Olianas *et al.* 2011[84]
		Rat NAc	
		NG108-15	
		NG108-15	Heis *et al.* 2009[85]
	↑ PI3K/ ↓ GSK-3β (SRC and AMPK dependent)	DOR-transfected CHO cells	Olianas *et al.* 2011[86]
	↑ PI3K (SRC and IGF-1 dependent)	DOR-transfected CHO cells	Olianas *et al.* 2011[87]
	↑ JNK (AKT dependent Pi3K mediated)	T cells	Shahabi *et al.* 2006[88]
ORL1	↑ ERK 1 and 2	Neuro-2a cells	Harrison *et al.* 2010[24]
		Rats NAc	Chen *et al.* 2008[89]
		In vivo (porcine)	Ross *et al.* 2005[90]
	↑ p38 MAPK	NG108-15	Zhang *et al.* 1999[91]
	↑ JNK	COS7 and NG108-15	Chan *et al.* 2000[92]
		In vivo	Ross *et al.* 2005[90]

↑ = activation; ↓ = deactivation; AKT = serine threonine protein kinase; AMPK = 5′ adenosine monophosphate-activated protein kinase; CHO = Chinese hamster ovary cells; CMT-93 = mouse rectum carcinoma cells; COS7 = monkey kidney fibroblast cell line; DOR = δ opioid receptors; EGFR = epidermal growth factor receptor; ERK1 and 2 = extracellular signal-regulated kinases 1 and 2; GRK-3 = G protein-receptor kinase 3; GSK = glycogen synthase kinase 3; HEK293 = human embryonic kidney cells; IGF-1 = insulin-like growth factor 1; IRF2 = interferon regulatory factor 2; JAK2 = Janus kinase 2; JNK 1 and 2 = c-jun N-terminal kinase; MAPK = p38 mitogen-activated protein kinase; NG108-15 = neuroblastoma glioma hybrid cell line; ORL1 = opioid receptor like-1; p38 STAT3 = signal transducer and activator transcription 3; PBMC = peripheral blood mononuclear cell; PI3K = phosphoinositide 3-kinase; SRC = proto-oncogene tyrosine-protein kinase.

In the past decades, various investigators have shown their efforts to find out possible mediating effects of OPRs against IR injury using different: pharmacological, ischemic and exercise preconditioning. DOP and KOP are strongly implicated in cardioprotection including anti-infarct and anti-arrhythmic actions across models and species (Table 3).

Table 3. Opioid Receptors Sub-Types and Function [94].

Receptor	Subtype	Function
MOP	μ_1	analgesia
	μ_2	analgesia, GI transit, respiratory depression, itching
	μ_3	various including NO release
DOP	δ_1	analgesia, cardioprotection
	δ_2	analgesia, cardioprotection, thermoregulation
KOP	κ_{1a}	analgesia, feeding
	κ_{1b}	
	κ_{2a}	analgesia, diuresis, neuroendocrine
	κ_{2b}	
	κ_3	spinal analgesia, peripheral effect

Opiate Receptor in Diabetes Mellitus

Type 2 diabetes is a common heterogeneous metabolic disorder which is influenced by genetic and several non-genetic factors including excess caloric intake and physical inactivity [95].According to the World Health Organization's estimation, more than 180 million people worldwide have diabetes mellitus (DM) [6]. This number is expected to increase more than double by 2030. Approximately 1.1 million people died from diabetes in 2005. Roughly half of diabetes deaths occur in people aged below 70 years and 55% of diabetes deaths are in women [6, 9]. There is a considerable increase in the incidence of this disease and it is widely recognized as a strong independent risk factor for coronary heart disease (CHD) especially among women [96]. Thirty percent of all patients undergoing aortic coronary artery bypass (CABG) are diabetic, these patients have post-operative mortality and mortality rates higher than the non-diabetic population with mortality rates of up to 50% to 90%. Myocardial infarction and Cardiac morbidity followed by stroke are among the common causes of late mortality after CABG in DM patients [97]. Compared to non-DM, these patients exhibit higher postoperative morbidity with higher percentages of higher re-interventions, decking, infections ,higher incidence of postoperative neurological complications and longer hospitalization periods [98, 99].It although reported that greater oxidative stress induced by cardiopulmonary

bypass (CPB) in DM patients than those without DM .In addition, both early and late mortality is certainly higher in DM patients[100]. Differences in the gene expression profiles of cardiac myocytes in DMP patients compared to non-diabetic NDMPs, particularly those related to inflammatory response and oxidative stress has been addressed as well as cardioprotective effects by opioid receptors [101].Hyperglycemia before and after cardiac surgery, in addition to inducing oxidative stress in the heart , contribution of coronary endothelial cells to cardiac adenosine production; It has been shown to cause mitochondrial dysfunction, cytochrome c release, and apoptosis .

Although, It has been reported that PKC, PI3 kinase/Akt, ERK1/2, STAT3, and GSK-3β phosphorylation impairment in diabetic hearts are the possible potential mechanisms that make the diabetic hearts more susceptible to IR and less sensitive to opioid conditioning [102].

However ,Mechanisms through which DM increases morbidity and mortality in myocardial revascularization and the role of opioid receptors on cardioprotection beside the multi functions of OPRs in cardiovascular physiology and neurotransmission, their expression in diabetic hearts, their cardioprotective roles in diabetes models of mice heart through Hdac inhibitor have not been evaluated yet. Thus, our study may contribute to better understand the important cardioprotective role of opioid receptors (particularly DOP, and KOP) in the STZ-induced diabetic mice model treated with HDAC inhibitor.

Histone Deacetylase Inhibition in I/R Injury

Histone deacetylases (HDAC) are a class of enzymes that remove acetyl groups (O=C-CH3) from a ε-N-acetyl lysine amino acid on a histone (or proteins) by the histone acetyltransferases (HATs) to allow the histones to wrap the DNA more tightly[103](Figure 6).This process is a vital aspect of epigenetic regulation of gene expression and more generally for the control of cellular stability that is regulated by acetylation and de-acetylation. In 1996 Taunton open the window to characterize the biochemical feature of the histone deacetylase after he succeed to clone and isolated HDAC1 for the first time[104].Indeed many article were published notably after this success. Today it is well known that HDACs play crucial roles in gene transcription and most likely in all eukaryotic biological processes that involve chromatin (Figure 7).

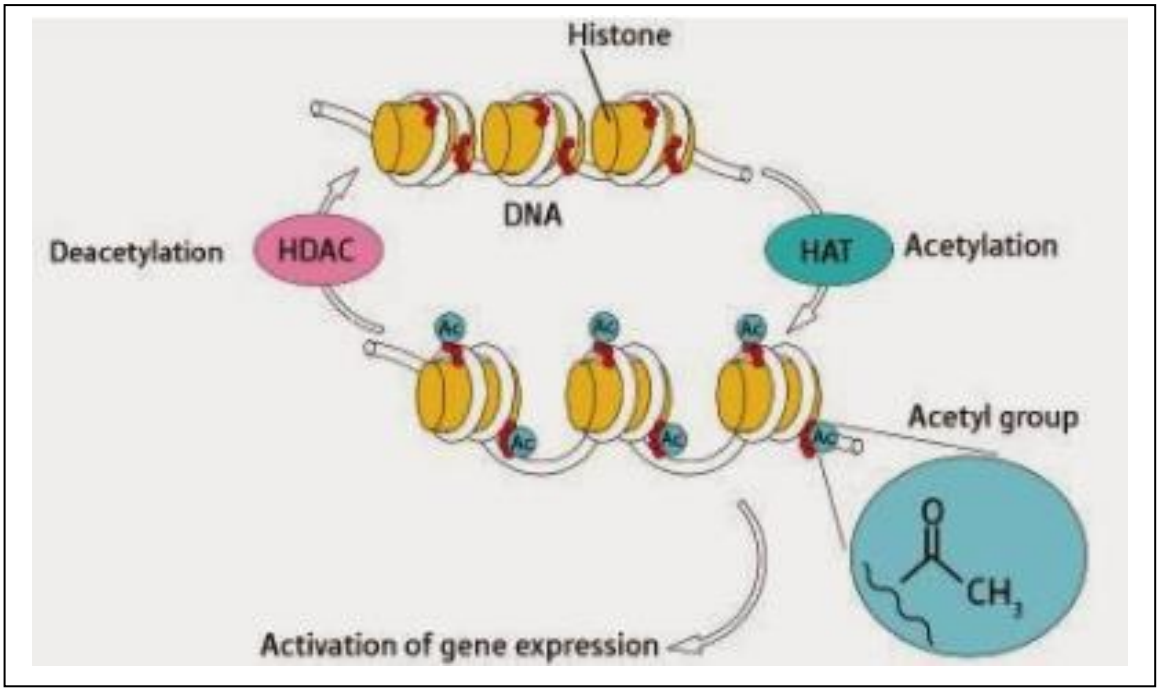


Figure 6 .Acetylation and Deacetylation. Key role for genes regulation [105].

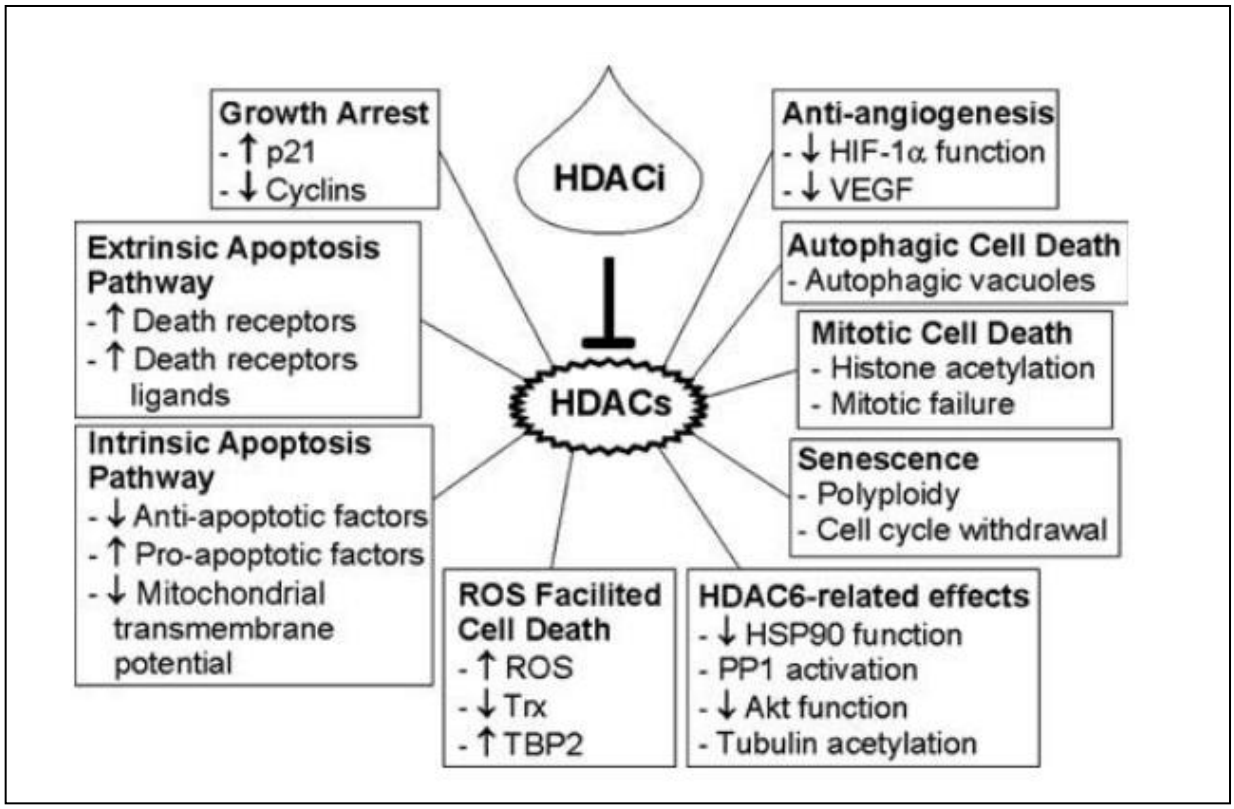


Figure 7. Multiple HDACi Pathways[106].

In mammalian cells, 18 HDACs have been described, grouped into 4 classes based on sequence homology and phylogenetic relationship [107]. Class I comprises HDACs 1, 2, 3, and 8, which

are located within the nucleus; class II comprises HDACs 4, 5, 6, 7, 9, and 10, which are located in both the nucleus and the cytoplasm; and class IV comprises HDAC 11 (Table 4)[108].HDACs also regulate the post-translational modification such as acetylating status of many non-histone proteins, including transcription factors, chaperones, and signaling molecules, resulting in changes in protein stability, protein-protein interactions, and protein-DNA interactions that regulate cell proliferation and cell death (Table 5) [109].

Table 4 .HDAC Classifications [110].

Superfamily	Family	Class	Protein (*S. cerevisiae*)	Subclass	Protein (human)
Arginase/deacetylase superfamily	Histone deacetylase family	Class I	Rpd3, Hos1, Hos2, Hos3		HDAC1, HDAC2, HDAC3, HDAC8
		Class II	Hda1	Class IIa	HDAC4, HDAC5, HDAC7, HDAC9
				Class IIb	HDAC6, HDAC10
		Class IV			HDAC11
Deoxyhypusine synthase like NAD/FAD-binding domain superfamily	Sir2 regulator family	Class III	Sir2, Hst1, Hst2, Hst3, Hst4	I	SIRT1, SIRT2, SIRT3
				II	SIRT4
				III	SIRT5
				IV	SIRT6, SIRT7

Table 5 .Non-Histone protein substrates of HDACs[106].

Function	*Proteins*
DNA binding transcriptional factors	p53, c-Myc, AML1, BCL-6, E2F1, E2F2, E2F3, GATA-1, GATA-2, GATA-3, GATA-4, Ying Yang 1 (YY1), NF-κB (RalA/p65), MEF2, CREB, HIF-1α, BETA2, POP-1, IRF-2, IRF-7, SRY, EKLF
Steroid receptors	Androgen receptor, estrogen receptor α, glucocorticoid receptor
Transcription coregulators	Rb, DEK, MSL-3, HMGI(Y)/HMGA1, CtBP2, PGC-1α
Signaling mediators	STAT3, Smad7, β-catenin, IRS-1
DNA repair enzymes	Ku70, WRN, TDG, NEIL2, FEN1
Nuclear import	Rch1, importin-α7
Chaperone protein	HSP90
Structural protein	α-Tubulin
Inflammation mediator	HMGB1
Viral proteins	E1A, L-HDAg, S-HDAg, T antigen, HIV Tat

So, it is not surprising that Epigenetic changes caused by imbalances between HATs and HDACs can affect global transcriptional profiles. In facts, HDAC knockout mice and gene deletion and/or overexpression studies have revealed important functions of several of these enzymes that are linked strongly with cardiovascular diseases, including coronary heart diseases [111], diabetic cardiomyopathy [112], hypertension [113, 114], ventricular remodeling [115, 116], and arrhythmia [117]. In addition, HDAC1 knockout mice have shown an embryonic lethal phase HDAC2 knockout mice are born alive but have severe cardiac defects and die within 24 hours [116].Conditional knockout of HDAC3 in cardiomyocytes leads to a dramatic upregulation of ligand-induced lipid storage within the heart. The mice survive for 3–4 months, at which point they show massive cardiac hypertrophy and depression of the genes that control fatty-acid uptake and metabolism [11]. HDAC5 and HDAC9 knockouts also have severe cardiac effects, including hypertrophy and fibrosis and were lethal with ventricular septal defects and a thin-walled myocardium (Table 6) [117].

They are extremely important in disease and now days are the target of many drugs. HDAC inhibitors (HDACi's) are common drugs for treating cancers, neurodegenerative diseases, and metabolic disorders to name a few [118]. HDACs inhibition results downstream changes in gene-expression promoting their therapeutic properties[119]. HDACi's have long been used in cancer treatments as well as in treating neurological condition. It has been reported that increased histone acetylation in the brain is associated with memory formation, while decreased acetylation reverse this effect [120, 121].

Table 6. Summaries of the various HDAC knockout phenotypes [122].

Isoforms	Phenotype	Related disease
HDAC1	Letheal (E10.5) Severe proliferation defects and generalgrowth re-tardation	
HDAC2	Die within 24h after birth Severe cardiac defect	Cardiac diseases
HDAC3	Lethal (before E9.5) owing to defects in gastrulation Deletion in the liver-disruption of lipid and choles-terol homeostasis Deletion in cardiomyocytes – massive cardiac hy-pertrophy	Cardiac diseases
HDAC4	Lethal within 7 days owing to ectopic ossification of endochondral cartilage, which prevents expansion of the rib cage and leads to an inability to breathe	Skeletal diseases
HDAC5	Viable Cardiac defects	Cardiac diseases
HDAC6	Viable but no obvious phenotype	
HDAC7	Embryonic lethality owing to a loss of integrity of endothelial-cell interactions and consequent rup-ture of blood vessels and haemorrhaging	Vascular disorders
HDAC8	Not determined	
HDAC9	Viable Cardiac defects	Cardiac diseases
HDAC10	Not determined	
HDAC11	Not determined	

They have demonstrated potent cardioprotective effect of a selective inhibition of classes I and II HDACs, trichostatin A (TSA) in murine models of myocardial ischemia/reperfusion (I/R),by reducing infarct size and preserved systolic function[123, 124] . This is in line with the observations that inhibition of HDACs in cardiac myocytes silences fetal gene activation, attenuates cardiac hypertrophy, and prevents cardiac remodeling [115, 125]. Also, TSA was recently reported to improve myocardial function and prevent cardiac remodeling in diabetic mice [126].

More interestingly, TSA giving at reperfusion only and after the ischemic insult still reduced infarct size to an extent similar to pretreatment [124, 125].These exciting results suggest that HDAC inhibition would be a novel drug strategies to patients presenting with myocardial infarction at the time of percutaneous coronary intervention in the cardiac catheterization laboratory. This idea has its own limitation because of large differences in the disease mechanisms in murine models and human case as previously reported. Based on this facts

,another HDAC inhibitor that is structurally similar to TSA, suberoylanilide hydroxamic acid (SAHA; vorinostat) (Figure 8),which is approved by the US Food and Drug Administration (FDA)for the treatment of cutaneous T-cell lymphoma, has been tested in a large-animal model of I/R by Min Xie (et colleagues 2014) and they have demonstrated a cardioprotective effect of SAHA ,by inducing prosurvival autophagy[127].

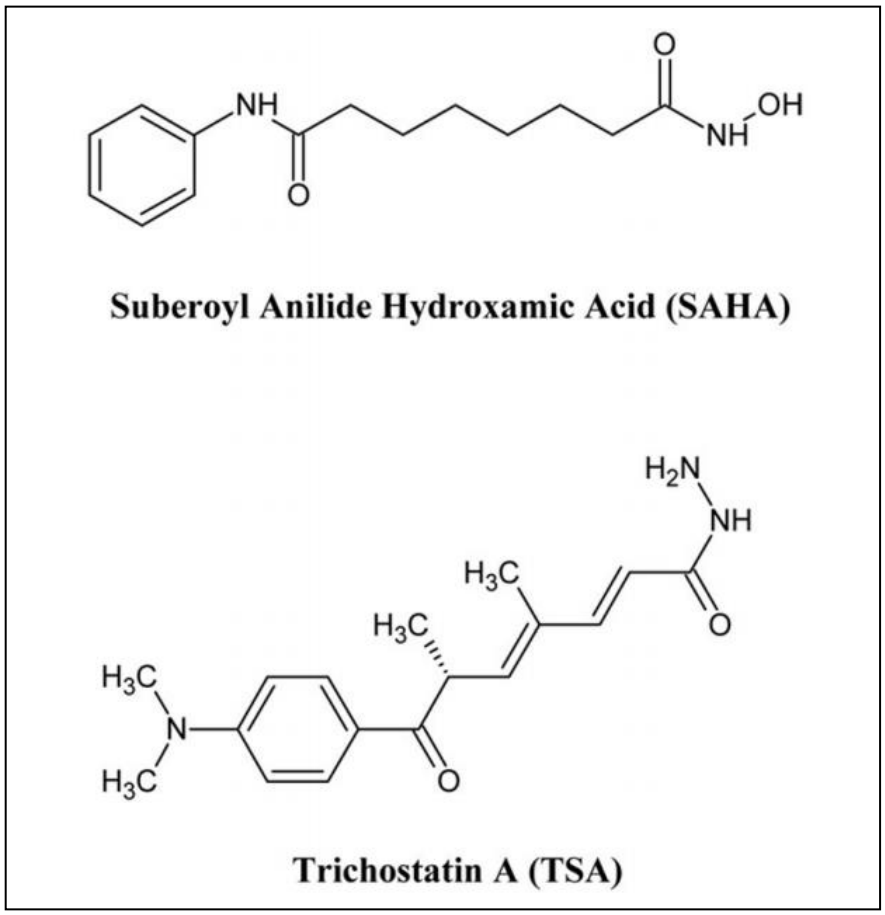


Figure 8. Chemical structure of SAHA and TSA[128].

Autophagy and Molecular Mechanism

Autophagy (self-eating) a well conserved dynamic process which is present in all cells. It results in the degradation of cytosolic components inside lysosomes in preparation for the turnover and recycling of cytoplasmic contents (e.g. proteins into amino acids or nucleic acids into nucleotides) [129]. Glick and colleagues have described three types of autophagy in mammalian cells, macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) respectively (Figure 9.) [130].

Macroautophagy is characterized by the delivery of cytoplasmic cargo to the lysosome through an intermediary double membrane-bound vesicle, known as an autophagosome, which fuses with the lysosome to form an autolysosome. Whereas, in microautophagy a direct engulfment of degraded contents through invagination of the lysosomal membrane [129, 130]. CMA

involves the direct translocation of cytoplasmic proteins across the lysosomal membrane in a complex with chaperone proteins receptor LAMP-2A (lysosomal-associated membrane protein 2A), resulting in their unfolding and degradation[131].

The processes of autophagy consist of three stages: Induction, autophagosome formation and fusion for degradation and recycling (Figure10).Briefly, in response to various stimuli, autophagy is induced by formation of a unique flat membrane (phagophore). The initiation requires two protein complexes involved in the regulation of autophagosome formation.The elongation of the phagophore results in the formation of an autophagosome, a double-membrane organelle. This step is a simple sequestration, and no degradation occurs.LC3B-II is found on both the inner and the outer surfaces of the autophagosome. During autophagy, the synthesis and processing of LC3 is increased and it is used as markers to monitor levels of autophagy in cells. Completely formed autophagosome are fused with the lysosomes in the cell. Autophagosome-lysosome fusion is mediated by the same machinery that is involved in homotypic vacuole membrane fusion [131-133].

The degradation of the vesicular cargo is dependent on a series of lysosomal/vacuolar acid hydrolysis. The resulting small molecules from the degradation, particularly amino acids, are transported back to the cytosol for protein synthesis and maintenance of cellular functions [134].

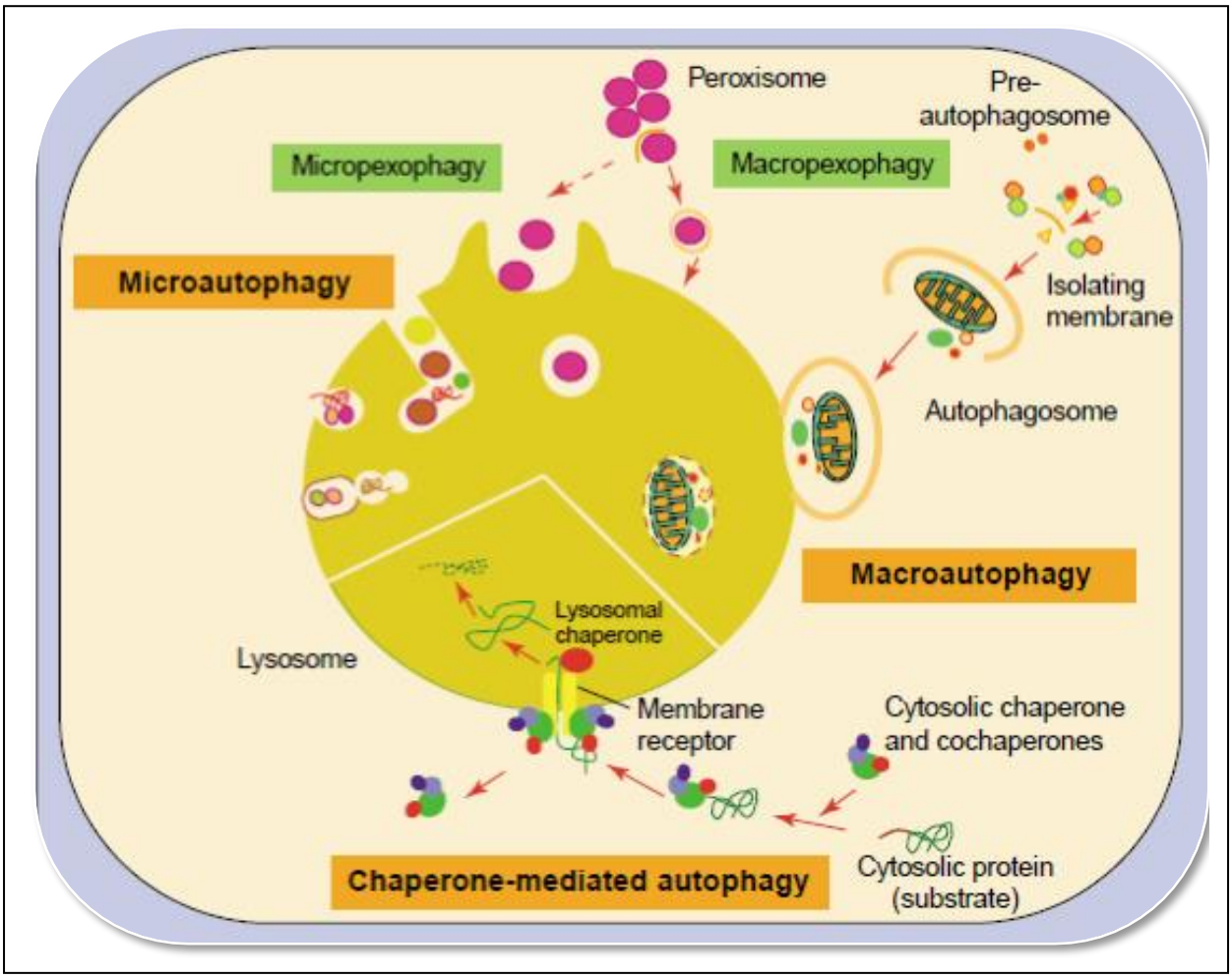


Figure 9. Types of autophagy in mammalian cells. Three main forms of autophagy exist: macroautophagy, microautophagy and chaperone-mediated autophagy. Internalized substrates could be different cytosolic organelles (circles) and/or single proteins [133].

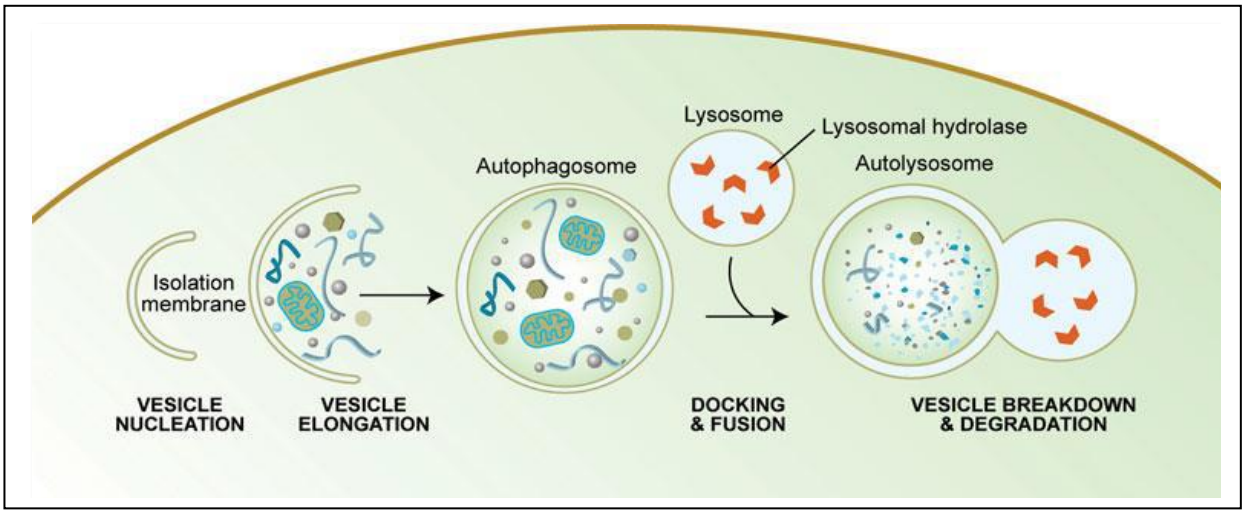


Figure 10.Steps of autophagy. Autophagy begins with the formation of the phagophore (vesicle nucleation step). Then, expansion of the phagophore into an autophagosome (vesicle elongation) taken place. Finally, the outer membrane of the autophagosome fuses with an endosome to form an autophagolysosome where, the sequestered material is degraded inside and recycled [135].

The Important Role of Autophagy

Many Genetic studies have emphasized the importance of autophagy in physiological and pathological events such as cancer, metabolic and neurodegenerative disorders as well as cardiovascular and pulmonary diseases. It also has been associated in aging and exercise in various organisms' models [136].

Autophagy at basal level has an important housekeeping role allowing cells to survive by supplying nutrient as shown in yeast and neonatal mice [137]. Also it has a role in cellular remodeling during differentiation and the development of multicellular organisms [138-141].Moreover, constitutive autophagy, which occurs independently of nutrient stress plays a key role in immune defense against invading bacteria and pathogens, and regulates inflammation, antigen presentation and micro-organism capture and degradation also, it contributes to liver homeostasis in animal model [141] (Table7).

It has been reported the protective role of autophagy in neurodegenerative diseases by removing unwanted cellular organelle and protein aggregates and in contrast, deletion of specific autophagy genes reverse this effect [142-147]. Although autophagy was linked to cancer, in a way to overcome nutrient-limiting conditions and facilitate tumor growth by promoting angiogenesis, supplying nutrients, and by modulating inflammatory response [148].

Table 7. Possible Autophagy outcomes in different pathologies [133].

Disease	Activation of autophagy	Inactivation of autophagy
Cancer		
Early stages	Blocks tumor growth	Favors tumor growth Makes cells unable to enter autophagic cell death after exposure to anticancer treatments
Late stages	Favors survival of cells in low-vascularized tumors Favors removal of damaged intracellular macromolecules after anticancer treatments	Prevents survival of cells in low-vascularized tumors Increases efficiency of anticancer treatments because damaged macromolecules cannot be eliminated
Vacuolar myopathies	Promotes elimination of the cytosolic autophagic vacuoles If hyperactivated, could result in muscle waste	Results in the accumulation of autophagic vacuoles that weaken skeletal and cardiac muscles
Neurodegeneration		
Early stages	Favors removal of cytosolic protein aggregates	Increases accumulation of cytosolic protein aggregates
Late stages	Destroys irreversibly damaged neurons by autophagic cell death	
Axonal injury	Favors removal of neurotransmitter vesicles and damaged organelles Provides energy and membranes for regeneration	Prevents removal of damaged organelles and neurotransmitter vesicles. Cytosolic release of neurotransmitters induces apoptosis Slows down regeneration
Infectious disease	Contributes to the elimination of bacterial and viral particles	Offers a survival environment for the bacteria that are able to inhibit autophagosome maturation Facilitates viral infection

Autophagy and Cardioprotection

The exponential growing of research has led to better understand the physiological functions attributed by autophagy and its role in many diseases. Besides that , when analyzing this role independent of the specific details of each disorder, a challenge is to determine whether autophagy protects or contributes to cell damage. In fact, depending on the intensity and duration of the insult autophagy could be a protective process or a cell death precursor therefore Autophagy termed as a double sward edges [149-151]. Perhaps, this is the reason for cardiology literature to pay a little attention to autophagy as a cellular protective mechanism. Here, we mainly focus on their protective effect in the heart. Recently ongoing research using experimental model and novel therapeutic drugs induce cardioprotective autophagy. It has been reported that autophagy occur during short periods of ischemia, however its contribution, for example, to preconditioning has not been studied yet. Classic autophagocytic vesicles and autophagy have been observed in the rabbit hearts made of hypoxia for 20-40 minutes and then reperfused and this is was associated with the functional recovery of myocytes [152, 153].

Min Xie and colleagues demonstrate that an FDA-approved HDAC inhibitor, SAHA, reduces myocardial infarct size in a rabbit-animal model by activation of cardiomyocyte autophagy, however the molecular mechanisms underlying this promising cardioprotective effects remain unknown. Thus our study may contribute to better understand the cardioprotective effect of autophagy in the heart.

Major Deficiencies in Our Present Knowledge and Hypotheses

Based on our introduction, the important role of opioid receptors to protect myocardium is an attractive field. Although a lot of studies have been done, still the role of opioid receptors is not well understood especially in the heart. With the knowledge that an FDA approved drug, SAHA, protects the heart during I/R in a rabbit large animal model by inducing pro-survival autophagy. It is not known whether Delta and Kappa opiate receptors expression levels are regulated by ischemia reperfusion and HDAC. Furthermore, it not known whether opiate receptor expression levels are changed in diabetic heart. Although we know Class I and II HDAC inhibitor, SAHA induces autophagy in the heart, it is not known which class of HDAC inhibitor is responsible for inducing autophagic flux in cardiomyocytes.

We hypothesize that HDAC inhibition Protects Cardiomyocytes by Modulating Expression of Opioid Receptor and Inducing Pro-survival Autophagy by Inhibiting Class I HDACs during Cardiac Ischemia/Reperfusion and opiate receptors is downregulated in diabetic heart.

This study was designed to evaluate by quantitate and qualitative approach the expression of opioid receptors and their mRNA levels, in vitro and in vivo wild type mice and STZ-induced diabetic mice (Figure 11) . Our study will lead to better understand the role of OPRs specifically with the treatment of an approved HDACi drug such as SAHA.

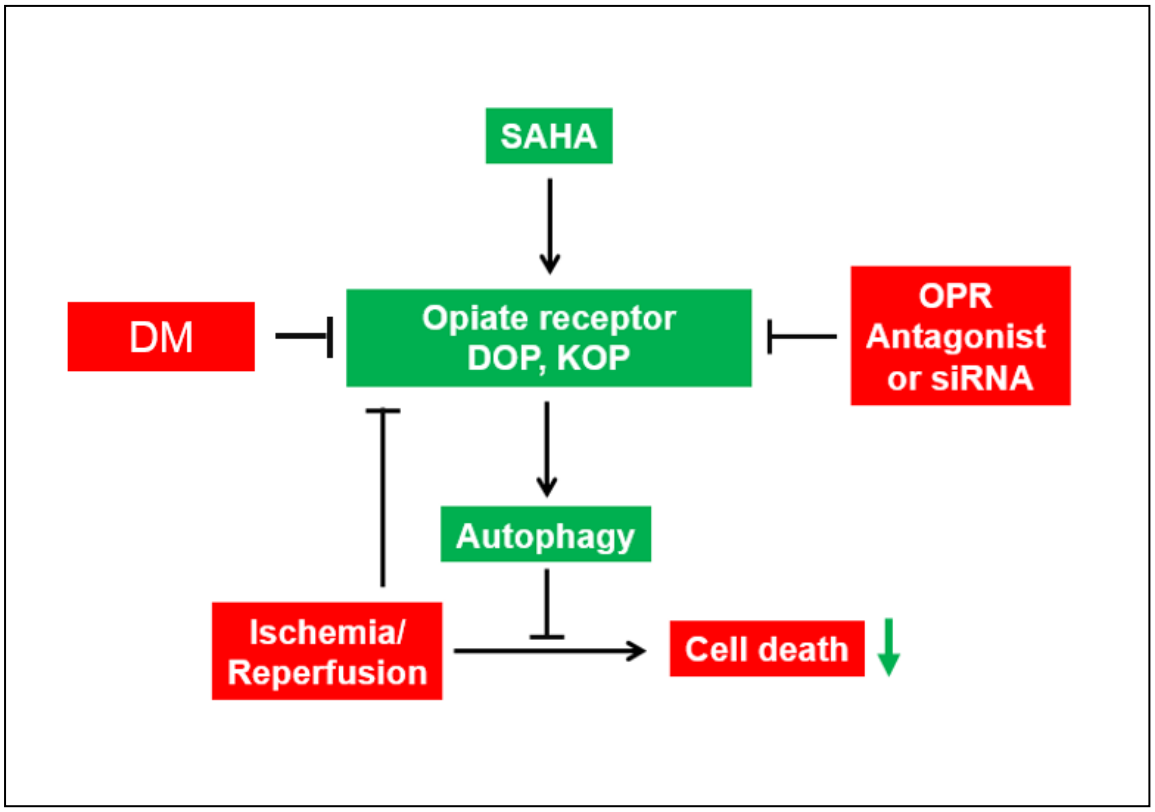


Figure.11 Flow chart of hypothesis.

Chapter II

Experimental procedures

Human immortalized ventricular myocytes Cell culture (AC16)

The AC16 cells were cultured in plate coated with 0.1% gelatin and incubated in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Hyclone, USA) with 10% fetal bovine serum (FBS) ,1.3% Hepes and 1% penicillin/streptomycin at 5% CO_2 and 37 °C up to80% confluence .Than AC16 cells were differentiated in HS 2% for 48 hours before starting their proper treatment.

Primary culture of Neonatal rat Ventricular myocytes (NRVM)

In brief, LVs from 1- to 2-day-old Sprague-Dawley rats were collectedand digested with collagenases. The resulting cell suspension was pre-plated to clear fibroblasts. Then the cells plated at a density of 1250 cells per 1 mm2 in medium containing 10% fetal bovine serum with 100 μmol/L bromodeoxyuridine. Typical cultures were notable for >95% cardiomyocytes. After overnight culture, medium with 10% horse serum was added for 24h, and then cultured in serum-free medium for 24 to 48 hours until further treatment.

Simulated I/R in Cultured Cells

For simulated ischemia/reperfusion (I/R) AC 16 or NRVM cell, ischemia was imposed by a buffer exchange to ischemia-mimetic solution (in mmol/L: 20 deoxyglucose, 125 NaCl, 8 KCl, 1.2 KH2PO4, 1.25 MgSO4, 1.2 CaCl2, 6.25 NaHCO3, 5 sodium lactate, 20 HEPES, pH 6.6) and placing the culture plates within a humidified gas chamber equilibrated with 95% N2, 5% CO2. After 2 hours of simulated ischemia, reperfusion was initiated by buffer exchange to normoxic AC16 culture medium or NRVM With HS2% OR 10% fetal bovine serum respectively, and incubation in 95% room air, 5% CO2. Controls incubated in normoxic culture medium for each kind of cells that were prepared in parallel for each condition.

siRNA knockdown in tissue culture

NRVMs were isolated and seeded at a density of 1.2 million/well in a 6-well dish. The purity of the cardiomyocytes is at least >85%. 24 hours after plating, cardiomyocytes were incubated with siRNA negative control (Neg, SIC001), siRNAs targeting DOP (SASI_Rn02_00259814), and siRNAs targeting KOP (SASI_Rn02_00261483), both from Sigma and used according to

the manufacturer's recommended protocols. Briefly, siRNAs were reconstituted into a 40 mM stock solution. 8µL of the siRNA stock and 8µL of RNAiMax transfectant were mixed together in 1 mL Optima medium. Cardiomyocytes were incubated with the RNAiMax for 6 hours, followed by addition of 1 mL of culture medium containing 20% serum. 24 hours after the siRNA incubation, the cardiomyocytes were treated with SAHA at 2µM (overnight). Then, the cells were subjected to ischemia (2 hours) and reperfusion (2 hours) for either immunoblotting or cells death assay.

Western blot analysis

Total proteins were extracted from myocardial tissues and Ac16 cells. Cells were lysed in 20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM glycerophosphate,1% Triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT), 2 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na3VO4. Protein concentration was measured using BCA assay .Forty µg were loaded on 12.5% SDS polyacrylamide gels and then transferred in wet transfer containing 25 mM Tris-base, 0.2 M Glycine, 20% Methanol, pH 8.5 for 2 hours in 450 mA into polyvinylidene difluoride (PVDF) membranes . The PVDF membranes were blocked with5% non-fat milk in TBS-T (0.1% Tween-20, AppliChem, Germany for 1 h. The primary antibodies such as Oprd1 (DOP), Oprk1 (KOP) (Santa Cruz biotechnology sc-9111, sc-7494) respectively, LC3I-II (Apg8b, Abgent San Diego, California, Us), P-62 (5114-Cell Signaling Technology, Danvers, US), and GAPDH (10R-2932-Fitzgerald, US) were incubated with agitation overnight at 4 °c. GAPDH was used as internal control. The secondary antibodies anti-goat (sc202-sigma), anti-mouse (Na931v,Ge Healthcare Bio-Sciences,Pittsburgh,US) secondary anti-rabbit (Na934v, Ge Healthcare Bio-Sciences,Pittsburgh,US) were incubated for another 1∼2 hours at RT after have been washed three times in TBS-T . Amersham imaging system (Ge Health Care, Marlborough, US) and ImageJ software was used to acquire and analyze the intensity of band respectively.

RNA isolation

Samples from myocardial tissues (~50mg) were homogenized with 1ml Trizol reagent (Invitrogen, Carlsbad, USA). Chloroform (200µl) was added to each Eppendorf tube and shacked vigorously by hands and then pre-incubated for 5 min at room temperature (RT) before centrifuged at 12, 000rpm for 15min at 4 ^{o}C. The upper supernatant was transferred to new Eppendorf tube .Isopropanol (500ul) was added to each tube and incubated at RT for 10min. Following 10min centrifugation at 12,000rpm, the supernatant was discarded and the pellet

was washed 2 times with 1 ml of 70% of ice cold ethanol. The ethanol was discarded and removed carefully by pipetting. After 10min air dry 20-50µl RNase-free water was added to suspend the RNA-pellet and incubated at 60^{o}C for 10min in a water bath. Finally, RNA concentration was measured by Nanodrop and stored at -80^{o}C until required.

Synthesis of cDNA

Complementary DNA (cDNA) were synthesized by using high-capacity cDNA reverse transcription kits (Applied Biosystems, Carlsbad, USA) according to the manufacturer's instructions. The following reagents were added to aPCR micro centrifuge tube on ice in the first mix: Total per reaction 10µL:10X RT Buffer 2µL , 25X dNTP Mix (100 mM) 0.8 µL, 10X RT Random Primers 2µL, MultiScribe™ Reverse Transcriptase 1µL, and Nuclease-free H2O 4.2 µL. The mixes were centrifuged gently and briefly .Then 10µL of RNA (250ng) sample were added, mixes well up and down. The contents were spinet down to eliminate any air bubbles before placing it in the thermal cycler at 25°C for 10minutes, 37°C for 120 minutes, 85°C for 5 minutes, and 4°C time to collect and were stored at -80°C until usage.

Quantitative Real-Time PCR

Real-time PCR (qPCR) was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems) using the SYBR Green PCR Master Mix Kit (Applied Biosystems, Carlsbad, USA) that contains all components except primers and cDNA template. Following primer mix for Oprd1 (DOP), Oprk1 (KOP) (SIGMA-Aldrich), relative expression levels of was determined. As an internal control GAPDH (Invitrogen, 059901, M5583 (A02, A01) was used. the data calculated by the delta-delta method as indicated previously [154].

Gene	Forward mouse (5`-3`)	Reverse mouse (5`-3`)
OPRD1	CGGTACACCAAATTGAAGAC	GTTGTAGTAGTCAATGGAGAG
OPRK1	AAAGTTGTGCCTCTATTGTG	TTGAAAACTGTCATGGTCTG
GAPDH	ATCAGCAATGCCTCCTGCAC	TGGTCATGAGTCCTTCCACG

Table4: Primer sequence used for real-time PCR (qPCR-RT).

Animals and experimental design

All animals were handled in this study in accordance with the standards established in the Guide for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animal Resources of the National Research Council (United States) and approved by the

Animal Care Committee of the University of Alabama at Birmingham. Wild-type male C57BL/6 mice used in this study were housed under identical conditions in a pathogen-free environment with a 12:12h light/dark cycle and free access to laboratory chow and water.

Mouse model of I/R

For I/R surgeries, 8 to 12-week-old C57BL/6 wild-type mice were utilized. C57BL/6 wild-type mice were anesthetized with 2-4% isoflurane and placed in a supine position on a heating pad (37°C). Animals were intubated with a 19G stump needle and ventilated with room air using a MiniVent mouse ventilator (Hugo Sachs Elektronik; stroke volume 250 µL, respiratory rate 210 breaths per minute). Following left thoracotomy between the second and third ribs, the LAD (Left Anterior Descending coronary artery) was visualized under a microscope and legated using a 6–0 prolene suture. Regional ischemia was confirmed by visual inspection under a dissecting microscope (Leica) of discoloration of the occluded distal myocardium. For I/R, the ligation was released after 45 minutes of ischemia and the tissue allowed to re-perfuse as confirmed by visual inspection.24 hours of reperfusion was performed after 45 minutes ischemia. Then the mice were sacrificed, the heart was extracted and tissue was used for subsequent Western blot, RNA isolation for further analyses.

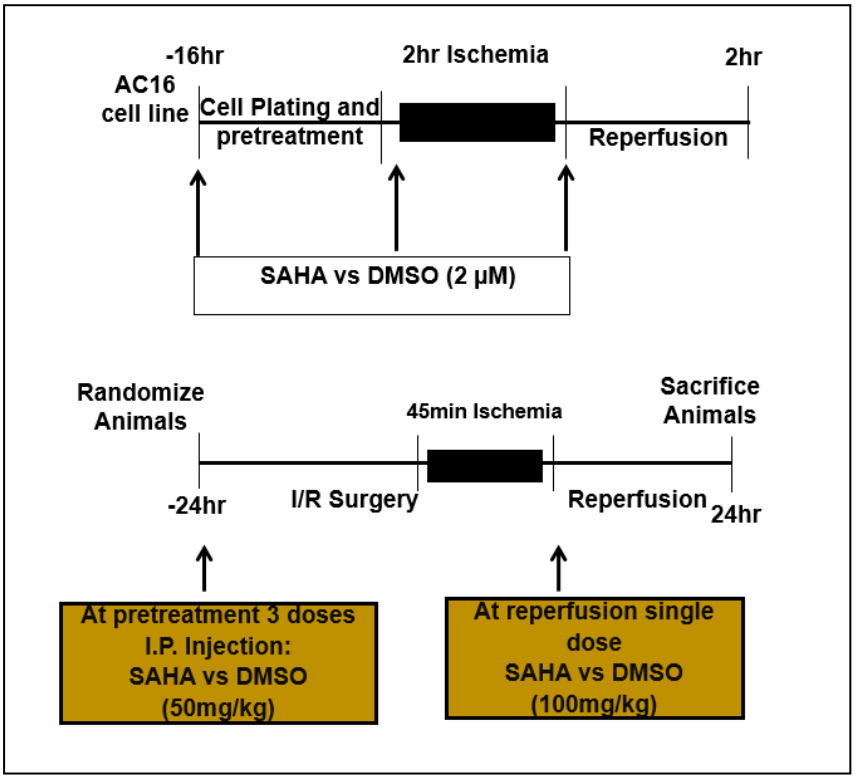


Figure 12. Simulated I/R Injury and mouse I/R Models.

Drugs preparation

SAHA and bafilomycin were purchased from LC Laboratory, were dissolved with DMSO to a final concentration of 2mM and 1mM respectively and aliquots in small tubes at -20c until further usages for cell treatment. SAHA injected to mice was freshly prepared [127]. Naloxone (E5008, D8147and product number from sigma were dissolved in sterile water (10mM).Streptozotocin (STZ) mixed anomers (STZ, product ref. S0130-500MG, Lot. No. WXBC2044V, SIGMA-Aldrich Chemie GmbH Kappelweg Schnelldorf, Germany) was dilute in 1ml of citrate buffer (10mM, pH 4.5) freshly prepared.

Diabetic mouse model

In STZ-induced model of diabetic mice prepared as previously described [155] .briefly, 8-12-week-old C57BL/6 wild-type mice were received IP injection of STZ dissolved in 10mM citrate buffer (45.5ml 0.1M citrate acid and 55.5ml 0.1M Na2HPO4, pH 4.5) at 40mg/kg after 4h fasting for 3 consecutive days. Mice were placed on high fat diet until the end of experiments. The survival rate was 100%.

Body weight and fasting blood glucose level measurement

Body weights of all groups of the rat were taken before STZ injection and after three and sixth weeks of IP injection. The blood collection site of the tail was wiped with 70% ethanol prior to place droplet of blood on a glucometer test strip. A blood sample obtained by pricking the lateral tail vein using a sterile needle and then the blood was gently milked from lateral tail vein and placed droplet of blood on a glucometer test strip and read using $^{\text{STATSTRIP}}$*Xpress-i* glucometer mg/dl (SN 138038215324, Nova Biomedical UK) and STAS-STRIP GLU SENSOR (Lot: 0315123309). Fasting blood glucose levels was measured after three and six weeks of STZ-induction. The mice in all groups were sacrificed after six weeks.

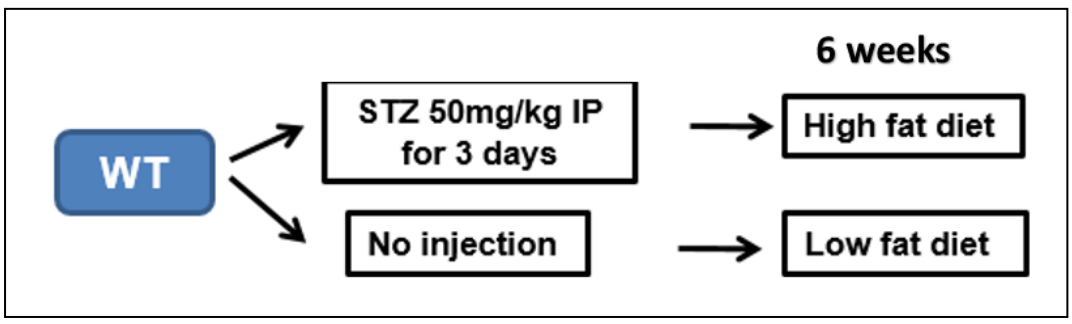


Figure.13 Type II diabetic mouse model.

Statistical analysis

Statistical analysis of the differences among groups was evaluated with a one way ANOVA followed by Duncan's multiple-comparison test using SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA). Significant differences were established at the level of $p < 0.05$. Data are expressed as means ±SEM.

Chapter III

RESULTS

PART-I Regulation of Opioid Receptors by I/R and SAHA.

OPRs are highly regulated receptors and has high expression level in in the heart [66, 67]. We are set to study the expression of OPRs in the setting of ischemia/reperfusion. Furthermore, we will look the effect of HDAC inhibition.

Opiate receptor expression is regulated by I/R.

In a vitro system of simulated I/R, we have evaluated the expression of OPRs in a time course manner (reperfusion for 30 min, 60 and 120min respectively) after 2h of ischemia. Simulated ischemia reduces DOP and KOP expression dramatically around 80 %, and their levels recover almost completely in 2 hours after reperfusion (N=3, P≤0.005) (Figure 14). Furthermore we have test whether autophagy is affected by I/R in Ac16 cell, by using baflomcycin (BFA), an autophagy inhibitor, and treatment for 2 hours. This blockage does not affect I/R regulated opiate receptor expression. However the trend of LC3II levels is similar to that of OPRs. During ischemia, autophagy measured by LC3II level is downregulated after ischemia ~ 80% and partially recovers during reperfusion for around 70 % (N=3, P≤0.005) (Figure 14). These indicate that autophagy and OPRs correlate to each other during cellular response to I/R injury.

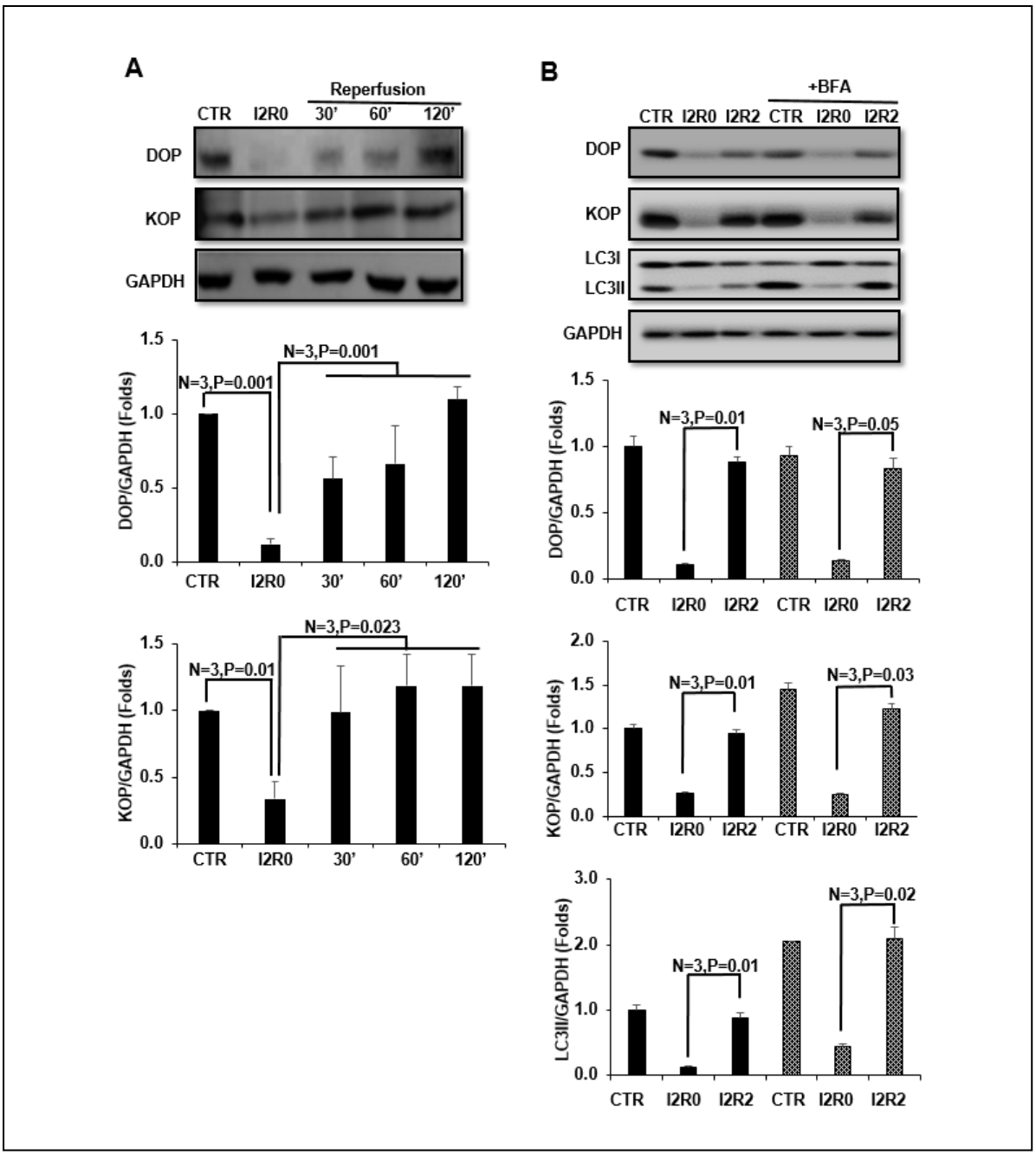


Figure 14. Opiate receptor expression is regulated by I/R. **A**. Simulated I/R reduces delta and kappa opiate receptor expression and their expression recovers soon after reperfusion. **B**. Block of autophagy does not affect I/R regulated opiate receptor expression and autophagic flux is downregulated after ischemia and recovers during reperfusion.

Opiate receptor expression is regulated by HDAC inhibition.

It has been reported that the protective effect of HDACi SAHA is dependent on inducing autophagy. Since autophagy and OPRs correlate to each other during cellular response to I/R injury, we decided to test whether SAHA treatment on AC16 cells to see whether SAHA will affect OPRs level. Indeed, SAHA increases DOP and KOP expression around 50% and maintains the autophagy flux two folds during I/R by Western blots ($N=3$, $P\leq0.005$) (Figure 15A). After getting this exciting data and keeping in mind the cardioprotective effect of SAHA in mouse, we treated wild type mice with SAHA injection as indicated in Figure 12. SAHA pretreatment for 24 hours increases DOP and KOP protein around 85% and mRNA expression 3.5 folds by Western blots and qRT-PCR($N=3$, $P\leq0.005$) (Figure 15B,C). Similarly, SAHA at reperfusion treatment only increases DOP and KOP expression around 65% by Western blots ($N=3$, $P\leq0.005$) (Figure 15D).

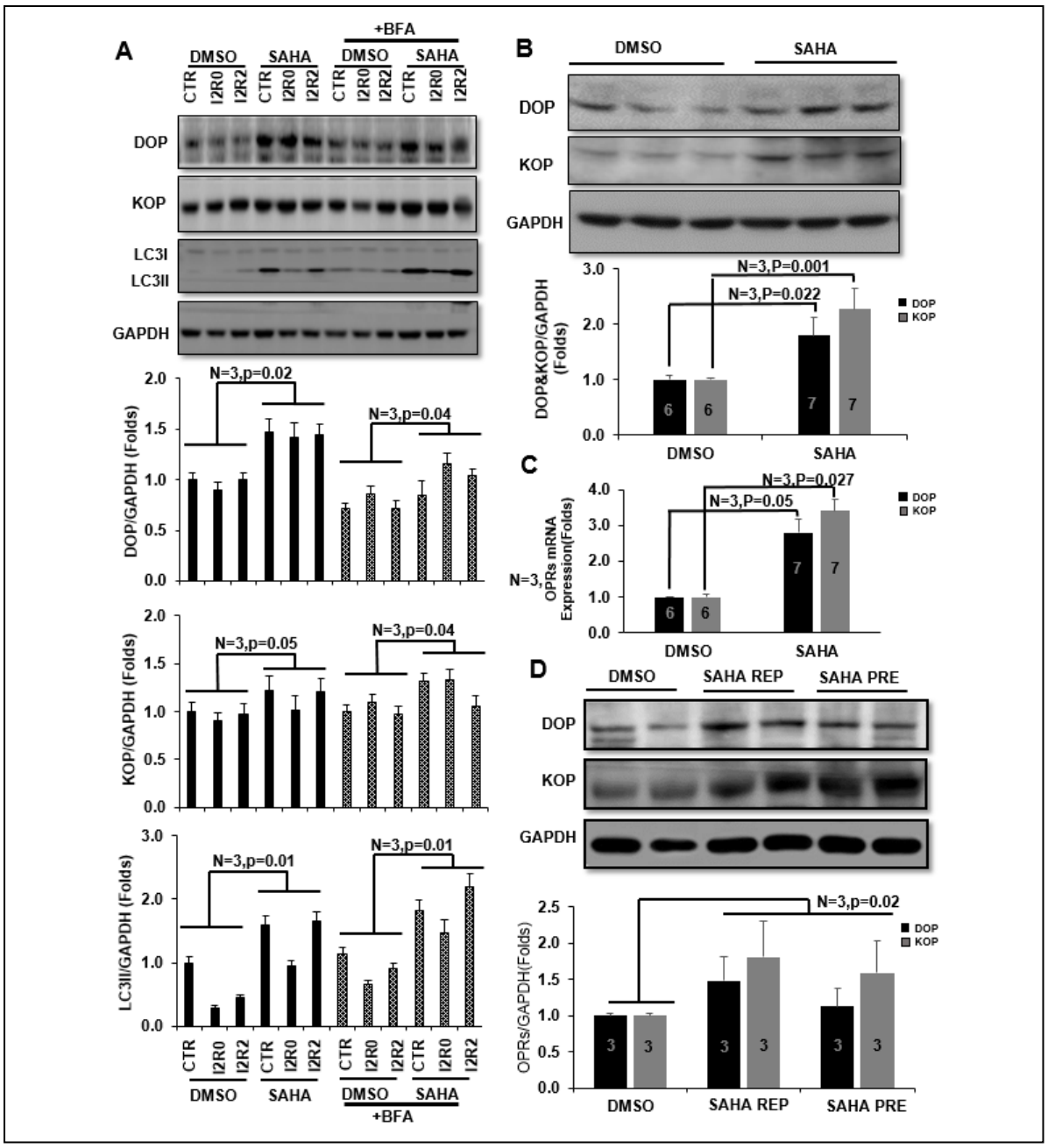


Figure 15. Opiate receptor expression is regulated by HDAC inhibition. A. In cultured AC16 cells, SAHA increases delta and kappa opiate receptor expression and maintain the autophagic flux during I/R by Western blots. **B**. In mouse heart, SAHA pretreatment for 24 hours increases delta and kappa opiate receptor expression by Western blots and **C**. qPCR. **D**. In mouse heart, SAHA reperfusion only treatment for 24 hours increases delta and kappa opiate receptor expression by Western blots.

It has been shown that SAHA reduces cell death during I/R. Based on this data, we moved on to test whether SAHA's cell protective effects are depended on OPRs activity. We have used Naloxone, an OPRs antagonist, to block their effect of OPR activation. We used LDH assay cell death assay as described in the methods. SAHA treatment reduces cell death after I/R by 15% (n-3, $p \leq 0.05$). In contrast, with naloxone treatment, SAHA failed to reduce cell death (Figure 16).

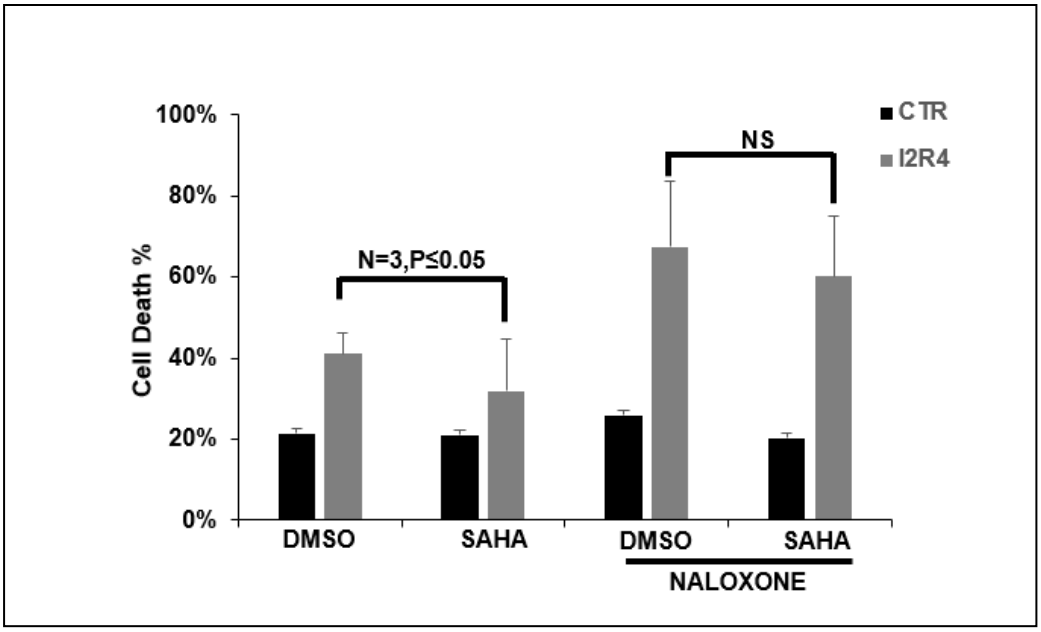


Figure 16. Opiate receptor expression blocked by Naloxone.LDH Cell death assay in cultured AC16 cells with the treatment of naloxone (OPRs-antagonist) and SAHA block opiate receptor expression and increase cell death.

PART- II Opiate receptor expression is downregulated in diabetic mouse hearts.

Diabetes has wide effects on the protein expression and function. We are set to test whether opiate receptor express levels are affected by the diabetic status. We have successfully generated a type II diabetic model using low dose STZ and high fat diet (Figure 17). The expression level of DOP and KOP protein and mRNA are downregulated around 50% in the diabetic mouse heart by Western blots and qRT-PCR (N=5, P≤0.005) respectively. The autophagy level is down-regulated around 63% in diabetic mouse heart (N=5, P≤0.005) (Figure 17). Then we tested the possibility for SAHA to rescue the reduced protein expression of OPRs in diabetic mice. STZ mice were injected with 2 dose of SAHA (Figure14). At mRNA level, both DOP and KOP are significantly higher with the treatment of SAHA (n=3, p≤0.05) (Figure 18-D).The expression of KOP and autophagy marker LC3II was significantly upregulated with SAHA compared to control group (n=3, p≤0.05). On contrast, SAHA treatment has no effect on the expression level of DOP and P62 (Figure 18).

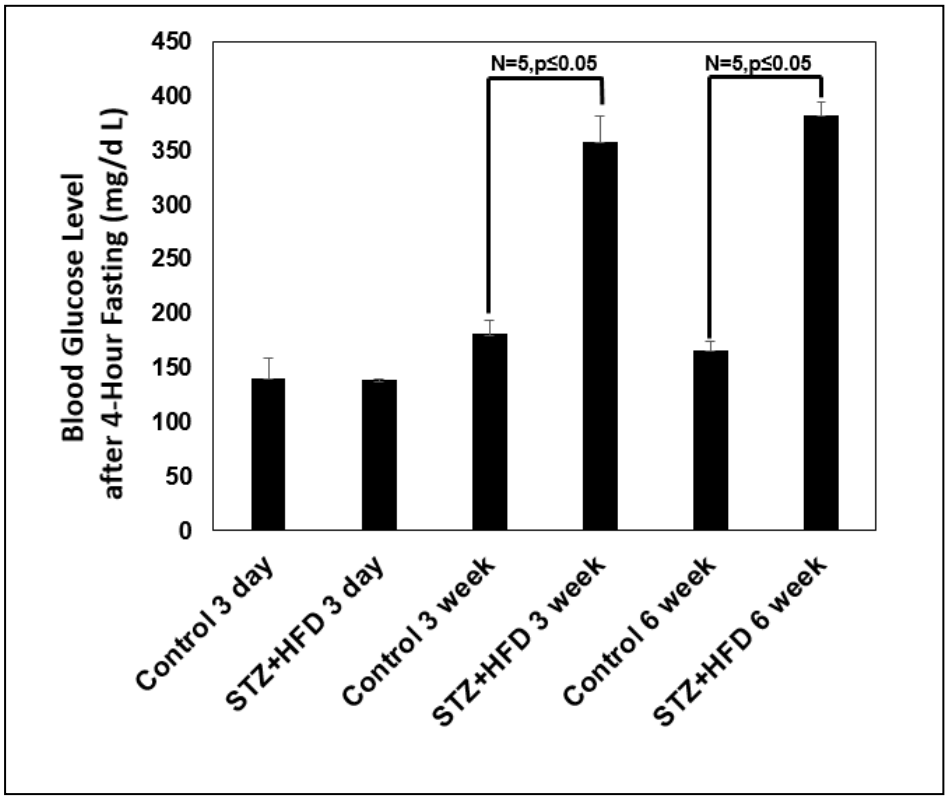


Figure 17. Blood glucose level in Type II mouse model.

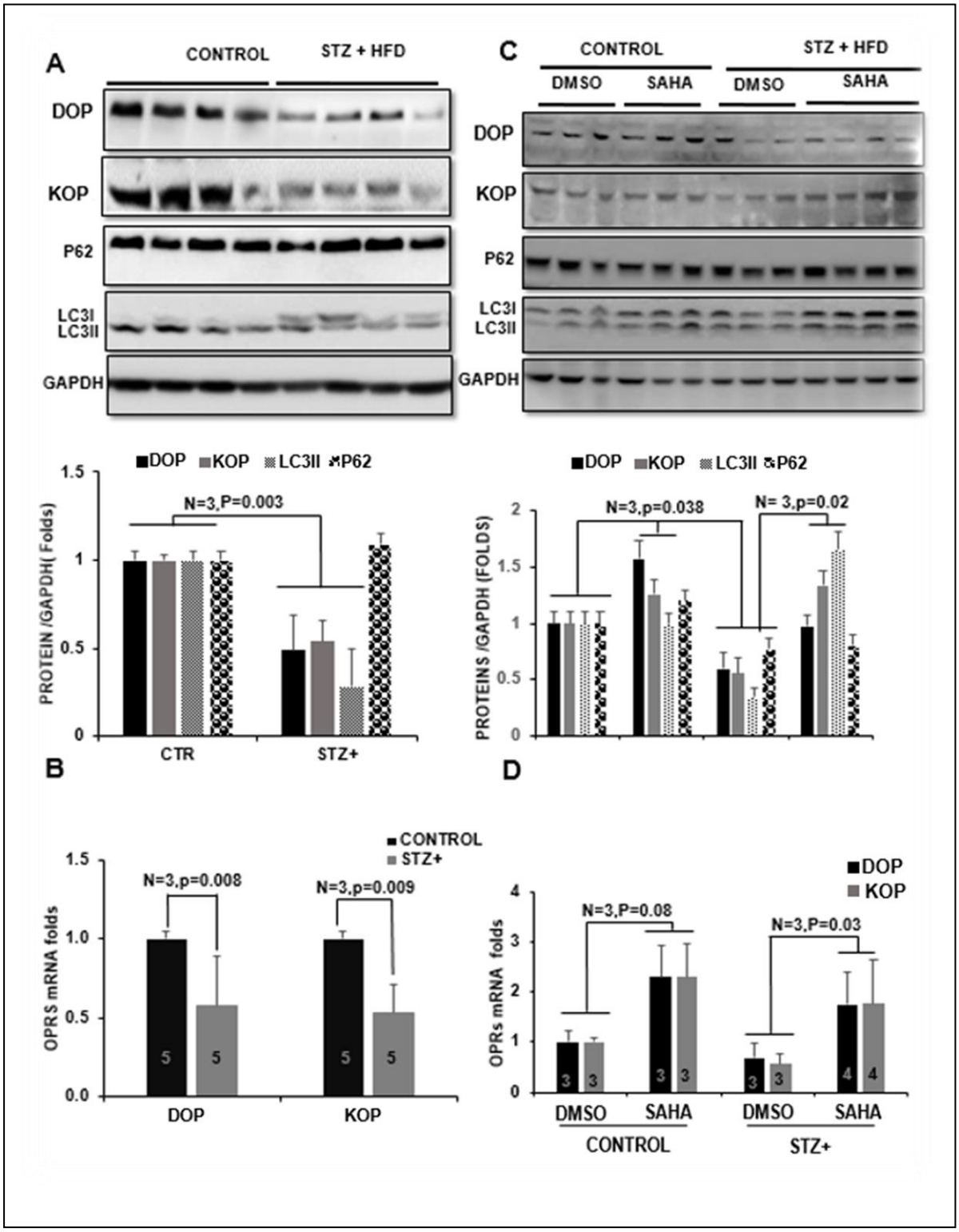


Figure 18. Opiate receptor expression is downregulated in diabetic mouse hearts. A. Expression of delta and kappa opiate receptors is downregulated in the mouse heart by Western blots and **B**. qRT-PCR. The autophagy level is downregulated in diabetic mouse heart. **C**.SAHA injection induce KOP receptor and autophagy significantly in diabetic mice. **D**. qRT-PCR.SAHA upregulate OPRs mRNA level is in diabetic mouse heart.

Part II HDAC Inhibition and Autophagic Flux in Cardiomyocytes

We know that the non-selective class I and II HDAC inhibitor, SAHA, induces autophagic flux in cardiomyocytes during I/R. However, we don't know which class of HDAC is responsible for regulating autophagy. In AC16 cells, Class I and II HDAC inhibitor, SAHA and Class I HDAC inhibitor, apicidin, induce two-folds autophagic flux (N=3, P≤0.005). While class II HDAC inhibitor, MC1568 did not induce autophagic flux.

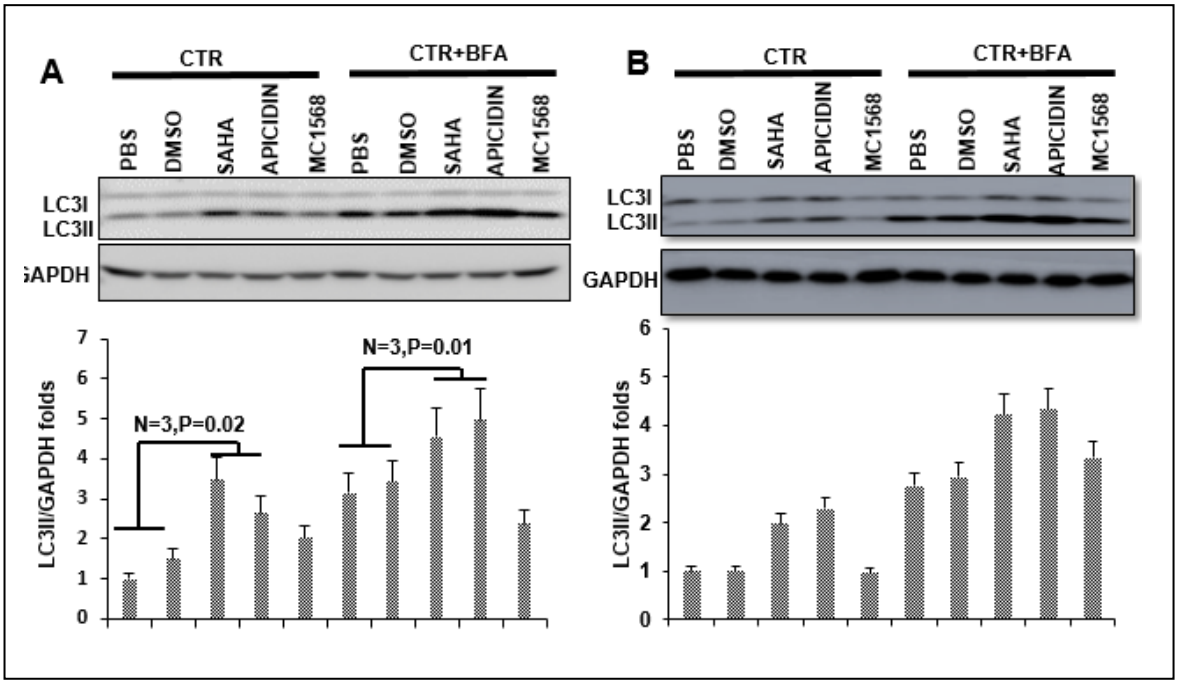


Figure 19. Class I HDAC inhibitor induces autophagy flux in cardiomyocytes.**A**.In Ac 16 cells, Class I and II .HDAC inhibitors,SAHA and class I HDAC inhibitor, Apicidin induce autophagic flux. While class II HDAC inhibitor, MC1568 does not induce autophagic flux.**B**.In human ES cell- derived cardiomyocytes (hES-CMs), we observed similar results (N=2).

Conclusions

Delta and Kappa opiate receptors expression levels are actively regulated by ischemia reperfusion. HDAC inhibition increases DOP and KOP expression. In diabetic heart, DOP and KOP expression are reduced. Class I HDAC inhibition induces autophagic flux in cardiomyocytes.

Chapter IV

Discussion

Patients have acute myocardial infarct and patients get on pump cardiac surgery have not only the risk of ischemic injury when the coronary artery is blocked or heart is arrested by cardioplegia solution, but also have subsequent reperfusion injury when the coronary artery is opened or when the heart is restarted. That is why it is very important to come up with novel strategies to protect the heart during this severe reperfusion injury. Novel therapies need to be developed to improve patient's quality of life, especially those with a contractile deficit profile seen before the surgery or large myocardial infarction. Targeting reperfusion injury has been studied for several decades and we still don't have a standard therapy in the clinical arena [16, 70]. Therefore, basic research and translational medicine are coming together try to find an effective therapies by applying experimental models that are as close to clinical reality as possible [14, 144, 155].Our experimental models used in this study are designed to mimicking human ischemic event during myocardial infarct in tissue culture and in mouse hearts.

Many studies have highlighted some cellular metabolic activity and proteomics profile that are changing during I/R [16-18] . Thus make them among the most interesting target to be investigated. OPRs are one of them as their protective effect have been elucidated through the availability of many drugs that can modulate their expression or activity especially during ischemic conditioning [13, 29, 39, 63, 71].

In our study, we have shown that I/R can modulate the expression of OPRs, specifically both receptor DOP and KOP were down regulated during a two hours of ischemia and tend to recover during the reperfusion in a time dependent fashion in our vitro system (80%). More interestingly, the autophagy profile was similar and goes down during Ischemia. Based on these data, we have hypothesized that OPR activation may cause autophagy. The autophagy during I/R is believed to be beneficial [127, 130, 134, 143, 147].The FDA approved anti-cancer HDACi, SAHA, has been shown to protect the myocardium in a large animal model by inducing autophagy and were able decrease the infarct size and maintain the heart physiology [127].That is why we thought that SAHA treatment may regulate OPRs expression and then subsequently autophagy. Indeed, in AC16 cell, SAHA pretreatment induces OPRs mRNA and protein levels, autophagic flux and reduces cell death. Moreover, in wild type mouse heart, pretreatment and reperfusion only treatment of SAHA upregulate the expression of opioid receptor at mRNA and protein level. To test the dependency of SAHA's cardiac protective

effects on OPRs, we used naloxone to block the activation of OPRs. We found that SAHA failed to reduce cell death during I/R with the presence of naloxone. Since naloxone as a chemical may have some unintended effects other than blocking OPRs, it might be helpful to test whether knocking down DOP or KOP using siRNA blocks SAHA's protective effects also. However, same as many in vitro studies, there are a lot of limitations of our AC16 cell system. We need to verify these results in bona fide cardiomyocytes such as neonatal rat ventricular myocytes (NRVM) or adult rat cardiomyocytes.

Since diabetes has worse clinical outcome during myocardial infarction [97, 101, 102]. we hypothesized that opioid receptors expression is downregulated. We generated a type II DM mouse model using low dose STZ and high fat diet. These mice have significant increased fasting glucose level. We have evaluated the expression of OPRs and was dramatically down in this model (50%) as well as autophagy. Furthermore we have injected SAHA to check whether it may restore the expression of OPRs and autophagy. SAHA injection increases the level of mRNA and upregulate autophagy and KOP significantly but not DOP. Ideally, these data need to be confirmed in human diabetic myocardium.

We also go on to check whether it is class I or class II HDACi that induce protective autophagy. We have used class I HDACi, apicidin and class II HDACi Mc1568. Apicidin increases autophagic flux up to 2 folds while Mc1568 did not. It will be interesting to see whether class I or class II HDACi has different effects on OPR expression. These results may lead to more specific autophagy inducers with less non-specific cytotoxic effects.

Chapter V: Significance and Future Directions

We have found that Delta and Kappa opiate receptors expression levels are actively regulated by ischemia reperfusion. HDAC inhibition increases DOP and KOP expression. In diabetic heart, DOP and KOP expression are reduced. Class I HDAC inhibition induces autophagic flux in cardiomyocytes. These results point out that opiate receptors are attractive therapeutic targets for reperfusion injury and agonists of the OPRs might be used in synergy with other cardiac protect medications such as adenosine and HDAC inhibitors. In diabetes, HDAC inhibition might be useful to restore the expression of OPRs and might increase the I/R tolerance in diabetes. Last but not least, we may use more specific class I HDAC inhibition to treat I/R injury to avoid the side effects of non-selective class I and II HDAC inhibition. As mentioned in the discussion, we will need to verify these findings in bona fide cardiomyocytes. We will also need to verify the downregulation of OPRs human diabetic heart samples. Furthermore, the mechanisms of how HDAC inhibition regulates OPR expression needs to be elucidated.

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