Chapter 11

Nitroso-Redox Crosstalk in Diabetic Cardiomyopathy

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Abstract

Diabetes mellitus is one of the most common chronic diseases worldwide. Diabetic cardiomyopathy (DM) is the deterioration of the myocardial function and morphology produced by the altered glucose metabolism imposed in diabetes. This process of cardiac deterioration involves the generation of oxidative species. In the diabetic heart, several sources contribute to the observed oxidative stress, such as xanthine oxidoreductase (XOR), nicotinamide adenine dinucleotide phosphate (NADPH), nitrogen oxidases (NOX), mitochondria, and uncoupled nitric oxide synthases (NOS). A direct consequence of the increased production of reactive oxygen species (ROS) is NOS uncoupling. This is the aftermath of the oxidation of tetrahydrobioterin (BH4), an essential cofactor for NOS activity. When NOS is uncoupled, its activity is redirected toward the production of superoxide, instead of nitric oxide (NO), further contributing to the oxidative process. This nitroso-redox disarrangement has a direct impact on the excitation-contraction-coupling machinery of the myocyte, in the mitochondrial stability impairing energy production and favoring apoptosis, myocardial fibrosis, ultimately reducing cardiac function. This review focuses on the impact of superoxide sources in the diabetic heart and the pharmacological approaches that are currently under investigation as possible therapeutic tools.

Keywords: Superoxide, nitric oxide, BH4, NOX, XOR

1. Introduction

Diabetes mellitus is one of the most common chronic diseases worldwide [1] and continues to increase in numbers and significance, with characteristics of an epidemic [2], as modern lifestyles lead to reduced physical activity and increased obesity.

Diabetic cardiomyopathy is the manifestation in the myocardium of the alterations produced by the altered homeostasis of glucose metabolism [3], independent of coronary artery disease and hypertension. This cardiomyopathy is characterized initially by diastolic dysfunction and cardiac hypertrophy, with preserved ejection fraction [4]. As diabetes progress, systolic dysfunction and reduced ejection fraction are developed [5]. This process of cardiac deterioration in diabetes includes oxidative stress [6] that results in apoptosis [7–9] and fibrosis that further deteriorate the myocardium. Despite the importance of diabetic cardiomyopathy as a clinical entity, the pathological cellular and molecular mechanisms driving the adverse changes in diabetic myocardial structure and function have not been fully resolved, but the development and progression of diabetic complications is frequently attributed to increased oxidative stress [6].

Reactive oxygen species (ROS) consist of a variety of highly reactive oxygen-based molecules that consist of both free radicals and chemicals capable of generating free radicals, such as superoxide, H_2O_2, and peroxynitrite ($ONOO^-$) [10]. Although in health the primary source of ROS is the mitochondria [11], they are also produced by a range of other sources such as xanthine oxidase, NADPH oxidases and uncoupled nitric oxide synthase (NOS). Oxidative stress takes place when the rate of production of ROS overrides the degradation by antioxidant enzymes. An increase in ROS levels leads to a constellation of harmful consequences by producing damage by oxidative modifications, diminishing of nitric oxide (NO) bioavailability, and by inducing alteration in the intracellular-signaling pathways [12]. In a variety of studies in animal models of diabetes and humans with diabetic cardiomyopathy, it has been shown that increased ROS levels [11, 13] have been associated with the pathophysiology of heart failure, including from cardiac remodeling and mechanical impairment [14]. This ROS-induced damage is probably a consequence of oxidative damage to cardiac proteins and also by inducing cell death in the myocardium [8, 13, 15].

In diabetes, the resultant hyperglycemia, hyperlipidemia, and insulin resistance enhance oxidative stress in the diabetic heart [16]. Altered glucose flux, mitochondrial dysfunction, and nitric oxide synthase uncoupling jeopardize the diabetic myocardium, increasing the risk of cardiac remodeling and the transition toward heart failure [3].

In the diabetic heart, the alterations in the excitation-contraction coupling machinery are profound [17]. The levels of the sarcoendoplasmic reticulum Ca^{2+} pump (SERCA) are reduced [18], and total phospholamban (PLB) is increased [19], while phosphorylated phospholamban is reduced. Phospholamban is a 5 kDa protein that tonically inhibits the activity of SERCA2. Upon activation, phospholamban is phosphorylated (by PKA or CamKII, for instance), oligomerizes, and releases SERCA of its inhibition [20]. In this condition, Ca^{2+} is transported back into the sarcoplasmic reticulum at a higher rate. The expression of the calcium release channel ryanodine receptor (RyR2) and the sodium-calcium exchanger (NCX) are reduced in general [18, 19, 21–24]. Functionally, this pattern of protein expression is associated with impaired relaxation, increased sarcoplasmic reticulum calcium leak, and reduced contractility.

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Altered calcium handling has been characterized in the diabetic cardiac myocytes [17]. Reduced capacity of the sarcoplasmic reticulum Ca^{2+} pump SERCA2 results in a diminished storage capacity of Ca^{2+} that impairs cardiac contractility [18, 19, 25]. Importantly, it also alters cardiac relaxation, which is evidenced in the diastolic dysfunction.

Oxidative stress may affect Ca^{2+} handling in the heart, at the level of the ryanodine receptor (RyR2) [26–28], which is the Ca^{2+} release channel of the sarcoplasmic reticulum. This channel is particularly redox sensitive and has been described that oxidative modification alters it function, leading to diastolic leak of Ca^{2+}, with a negative impact of contractility and relaxation [28–30].

In diabetes, several sources may contribute to the observed oxidative stress, such as xanthine oxidoreductase (XOR) [31, 32], NADPH oxidases (NOX) [33–35], mitochondria [11], and uncoupled nitric oxide synthases (NOS) [36, 37]. Apparently, the main source of superoxide in the diabetic heart corresponds to XOR and NOX. These latter enzymes are present in the heart, mainly with the isoforms NOX2 and NOX4.

A direct consequence of the increased production of ROS is the uncoupling of nitric oxide synthase [38]. This is due to the oxidation of tetrahydrobioterin, an essential cofactor for NOS activity [39]. When NOS is uncoupled, its activity is redirected toward the production of superoxide, instead of NO [26], further contributing to the oxidative process.

2. NADPH oxidases NOX

Nicotinamide adenine dinucleotide phosphate oxidase enzymes (NADPH oxidases or NOX) received growing attention as a source of ROS and particularly for their involvement in redox signaling [40]. NOX exist in several isoforms (NOX1, 2, 4, and 5) [41]. These enzymes catalyze for electron transfer from NADPH to molecular oxygen, resulting in the generation of oxygen-free radicals. NADPH oxidase is increased in both failing and diabetic rodent hearts [42–44]. In diabetic rodents, the upregulation of NADPH oxidase correlates with morphological evidence of cardiac hypertrophy and upregulation of pro-fibrotic genes [42, 43, 45].

NOX2 is formed by the membrane-associated $p22^{phox}$ and one $gp91^{phox}$. The translocation of the cytosolic regulatory subunits $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and the small G protein Rac1 (the isoform expressed in the cardiomyocyte) or Rac2 is necessary for the occurrence of electron transfer from NADPH to oxygen. Under stimulation, for instance, with high levels of glucose or AngII, PKC is activated, leading to phosphorylation of $p47^{phox}$, an event that promotes the association of cytosolic subunits with p22phox and gp91phox [41] (**Figure 1**). In the cardiovascular system, these enzymatic components are expressed in endothelial cells, smooth muscle, cardiomyocytes, and fibroblasts [41, 46].

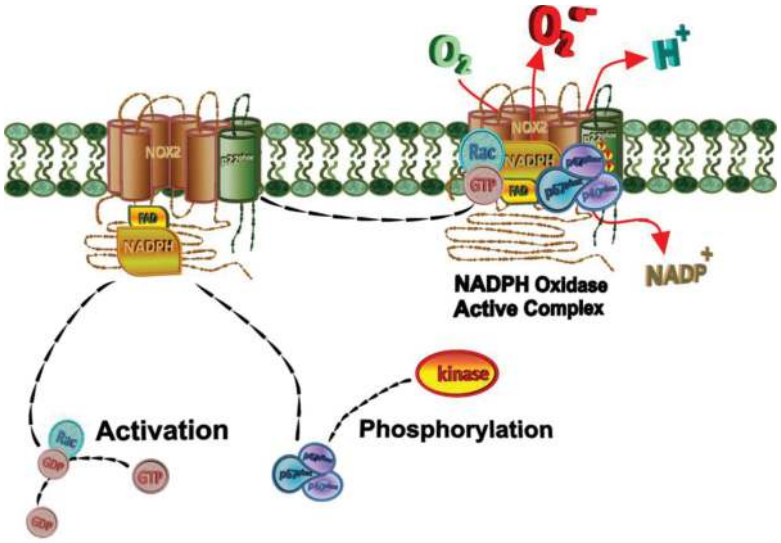

Figure 1. Mechanism of NADPH oxidase activation. Upon proper stimulus, Rac is activated and $p47^{phox}$ is phosphorylated. Then $p47^{phox}$ binds $p40^{phox}$ and $p67^{phox}$ and along with Rac, translocate to the cell membrane, where they activate NOX2 superoxide production.

2.1. NOX in diabetes

Several reports indicate that an increased activity of NOX, particularly NOX2 [34, 43, 47, 48] and NOX4 [35], may be implicated in the pathophysiology of diabetic cardiomyopathy. Hyperglycemia induces O_{2-} generation in the heart primarily by disruption of the electron transport chain in mitochondria, activation of NOX and uncoupling of NOS. In type-2 diabetes, a state of permanent oxidative stress alters mitochondrial function, impairing energy production and ultimately producing cardiomyocyte dysfunction. Elevated glucose levels have been shown to stimulate NOX activity, increasing NOX-derived superoxide production in cardiac cells [48].

2.2. Physiological functions of NOX2

Recently, Prosser *et al*. elegantly demonstrated a physiological role for NOX2 in the cardiac myocytes, demonstrating that NOX2 activity is required to stimulate RyR2 in conditions of physiological stretching (preload). NOX2 activation upon stretching sensitizes RyR2 increasing the rate of Ca^{2+} sparks [49]. This mechanism becomes dysregulated in dystrophic cardiomyopathy [50]. Furthermore, more recently, it has been published that NOX2 in conjunction with NOS1 are absolutely required for another cardiac physiological response to an increase in afterload (Anrep effect) [51]. NOX2 oxidase has been implicated in the cardiac dysfunction observed in ob/ob cardiac myocytes [34, 52].

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2.3. NOX2 and diabetic cardiomyopathy

Several lines of evidence showed a role of NOX2 in the diabetic cardiomyopathy [53, 54]. First, NOX2 is activated and its subunits are upregulated in several models of diabetes and insulin resistance, streptozotocin-treated mice, ob/ob and db/db mice, and fructose-fed rats, with increased oxidative stress as a consequence.

Using streptozotocin-treated mice, Roe *et al*. [55] described that pharmacological inhibition of NADPH oxidase with apocynin reduced part of the cardiac damage and it improved heart functional evaluated as fractional shortening, and cardiomyocytes mechanics, associated with a reduction in nitrosative and oxidative stress. Nevertheless, since apocynin is not a specific inhibitor for NOX2, these results might be ascribed also to NOX4, which is also present in the cardiomyocyte.

Evidence for a more specific and crucial role for NOX2 came from studies using knock out for Rac1, one of the cytosolic components of the holoenzyme complex. In cardiac-specific Rac-1 knockout mice, induction of diabetes by streptozotocin treatment showed reduced apoptosis, fibrosis and improved cardiac function compared to wild-type animals [56, 57]. The mechanisms of reduced cardiac damage involves a reduction in NADPH oxidase activity, a reduction in mitochondrial ROS production that attenuates fetal gene program, reduces the inflammatory process, and reduces endoplasmic reticulum stress. In addition, cardiomyocyte-specific deletion of Rac-1 reduces cardiac damage in diabetic mice after ischemia-reperfusion by reducing calpain proteolictic activity [58].

2.4. NOX4

NOX4 is expressed constitutively in the heart and its activity has been ascribed as mitochondrial [59]. Its physiological role has not been clarified. On the contrary, there are descriptions of NOX4 in pathological conditions that are both beneficial [60] and deleterious [44]. Indeed, Sadoshima's group has shown that NOX4 is an important source of oxidative stress in a mouse model of pressure overload (by aortic constriction). In this study, NOX4 deletion was associated with preserved ejection fraction and reduced hypertrophy, apoptosis, and mitochondrial function compared to wild-type animals [44]. Nevertheless, these results are contrary to those observed by Shah's group. These investigators reported a protective role for NOX4, using the same strategy (pressure overload in a cardiomyocyte-specific knock-out mouse for NOX4), with the difference that they additionally used a mouse with cardiomyocyte-targeted increase in NOX4, which showed an increased angiogenesis in response to overload [60].

Regarding diabetic cardiomyopathy, Maalouf *et al.* reported that NOX4 plays a pivotal role in the cardiac damage in streptozotocin-treated rats. They found that NOX4 was up upregulated and when silenced using antisense oligonucleotides, this was reversed, along with the induction of fetal program genes and fibrosis, as well as cardiac function [35].

3. Xanthine oxidoreductase

Xanthine oxidoreductase (XOR) is a highly conserved member of the molybdoenzyme family. It consists of a 150 kDa homodimer that is involved in the purine degradation pathway, producing superoxide as a secondary metabolite. This precursor may further divert in the formation of two enzymatic activities, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). XO is derived from XDH as a result of proteolisis or reversible oxidation of cysteines of XDH. Whereas XDH reduces NAD^+ to NADH, XO utilizes molecular oxygen as electron acceptor. They differ in that XO only reduces oxygen, whereas XDH can reduce either O_2 or NAD^+. Both forms catalyze the conversion of hypoxanthine to xanthine and xanthine to uric acid. In both steps, O_{2-} and H_2O_2 are produced [61]. Normally, in the cardiac myocyte, XOR is located in the sarcoplasmic reticulum, where it regulates some features of calcium handling and also myofilament calcium sensitivity. We have reported that XOR is upregulated in dilated cardiomyopathy, producing oxidative stress that interferes with NO signaling, resulting in dysregulated RyR2 activity, ultimately producing diastolic calcium leak and reducing contractility [28]. It is probably that in diabetes, XOR may impact negatively calcium handling, but this has not been clarified.

XOR has been implicated in the pathophysiology of streptozotocin-induced diabetic cardiomyopathy and its inhibition with allopurinol, oxypurinol, and antioxidants yielded some positive results that included improvement in cardiac function, reduction in oxidative stress, reduced cardiac apoptosis, fibrosis (collagen I), and hypertrophy [31, 33, 62]. In obese (ob/ob mice), XOR is upregulated and its activity increased, leading to alterations in the redox state of the heart, such as diminished GSH/GSSG ratio and decreased levels of NO metabolites [32].

Importantly, XOR has been shown to be increased in type-1 diabetic patients [63]. In addition, in a study in patients with type-2 diabetes and left ventricular hypertrophy, the treatment with allopurinol reduced left ventricular mass modestly but significantly in a period of 9 months of treatment, in a dosage that reduced plasma uric acid by ~50% [64].

4. Mitochondria

Due to their high-energy demand, cardiomyocytes are abundant in mitochondria. However, mitochondria are also an important source of ROS and NO [65, 66].

In physiological conditions, most of the reducing equivalents of the electron transport chain are directed toward the production of ATP and about 0.1% leak and produces superoxide [11].

Diabetes induces a series of metabolic derangements in myocardial mitochondria [12]. Under conditions of hyperglycemia, the mitochondrial inner membrane hyperpolarizes and inhibits the electron transport in complex III, generating superoxide [67].

Myocardial diabetic damage is reduced by overexpression of Mn SOD and catalase [57, 68] and by pharmacological inhibition of mitochondrial ROS by a mitochondrial-directed SOD

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mimetic, mito-Tempo [69]. Noteworthy, besides being a source of ROS, mitochondria may suffer the consequences of increase oxidative stress as well [70]. High levels of ROS may sensitize the mitochondrial permeability transition pore (mPTP), releasing cytochrome C, and Smac/DIABLO ultimately inducing apoptosis [71]. The mitochondrial permeability transition pore is constituted by the adenine nucleotide translocase (ANT), the voltage-dependent anionic channel (VDAC), and cyclophilin D, which under stress conditions such as high Ca^{2+} or increased ROS make the mitochondrial membrane permeable to ions and molecules <1500 Da, dissipating the mitochondrial membrane potential [72]. In human atrial tissue from patients with diabetes, Anderson *et al*. [73] found increased H_2O_2 production, with increased sensitivity to Ca^{2+}-induced mPTP opening compared with nondiabetic patients and also in the heart of Zucker rats [74].

The components of the mPTP contain several redox-sensitive sites that can be oxidized or *S*-nitrosylated [71, 72]. *S*-Nitrosylation generally prevents mPTP opening, while oxidation sensitizes to opening (**Figure 2**). This is consistent with the fact that NO donors, at low concentrations, protect the mPTP from opening [72, 75, 76]. For instance, cyclophilin D has been shown to undergo *S*-nitrosylation in cysteine 203, preventing the oxidative-induced opening of the pore [77]. High NO donor concentrations, on the other, promote mPTP opening, associated with oxidation rather than hypernytrosylation. Nevertheless, the role of *S*-nitrosylation of mPTP in conditions of diabetic cardiomyopathy remains to be determined.

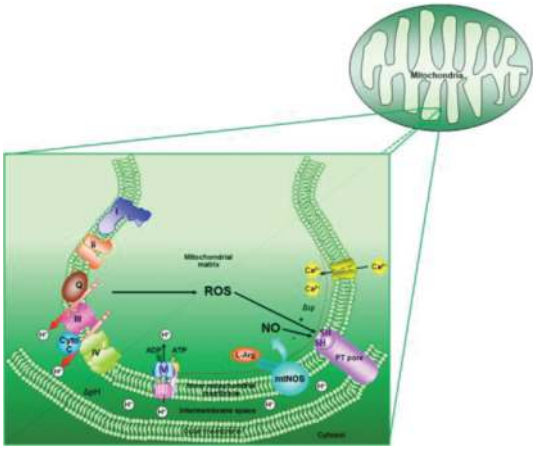

Figure 2. Potential role for nitric oxide in the mitochondrial permeability transition pore (PT). Nitric oxide produced within the mitochondria, probably by a mtNOS, reacts with sulfhydrils groups in the pore, *S*-nitrosylating them and preventing oxidation by ROS. This prevents the pore from opening and dissipating mitochondrial protons gradient.

In addition, mitochondria appear to play a central role in diabetic cardiomyopathy through alterations in the process of authopagie, and this process is clearly influenced by the mitochondrial oxidative status [15, 78–81].

5. Nitric oxide

Nitric oxide (NO) is a free radical that is produced by a family of NO synthases (NOSs), using NADPH, the amino acid L-arginine and oxygen as substrates. NOSs are homodimers and each subunit contains one flavin mononucleotide (FMN), one flavin adenine dinucleotide , one tetrahydrobiopterin (BH_4), and one Fe(3)-heme cofactor that facilitates the 5-electron oxidation of L-arginine to yield NO. In biological systems, NO exerts it effects by redox reactions (such as *S*-nitrosylation) or by inducing the production of cGMP as a second messenger [27, 82, 83].

Nitric oxide plays several important roles in the physiology of the myocardium, both in cardiomyocytes and in blood vessels [83–85]. NOS1 (former neuronal NOS) and eNOS (or NOS3) are expressed constitutively in the heart structures, particularly in the cardiomyocyte, NOS1 participates in the regulation of contractility [86, 87] by regulating RyR2 *S*-nitrosylation [29, 30] and phospholamban phosphorylation [88, 89]. NOS1 regulates the β-adrenergic [86, 90] and force-frequency response [29, 91] in the heart. Also NOS1 prevents cardiac dysfunction after a myocardial infarction and pressure overload [92–95]. For these reasons, the role for NO in diabetic cardiomyopathy has gained attention [96–98].

5.1. NOS1

Our group has recently identified specific stimulatory influences of NOS1 on both β-adrenergic inotropic responses and Ca^{2+} transients ($[Ca^{2+}]_i$) using $NOS1^{-/-}$ mice. These results are consistent with *in vitro* studies indicating that NO regulates SR Ca^{2+} release via the RyR by *S*-nitrosylation of a specific thiol moiety. RyR2 activation via nitrosylation is highly sensitive to ROS. We demonstrated non-cGMP related, redox-sensitive positive inotropic effects in isolated heart preparations [82] and demonstrated the role of NOS1 specifically in SR Ca^{2+} cycling [29, 99]. The interplay between NO and ROS is also of critical importance for Ca^{2+} handling. In the SR, NO augments RyR activity via *S*-nitrosylation in a manner regulated by ROS [100, 101]. ROS also activates the RyR2 but promotes maximal channel activity in an irreversible manner, reducing the ability of NO to exert feedback regulation of SR Ca^{2+} release. Such a situation is likely to lead to an increased Ca^{2+} leak through the SR and "futile" Ca^{2+} cycling, which increases ATP expenditure. It is also conceivable that oxidant signaling may directly regulate cross-bridge cycling kinetics, thereby modulating the efficiency of contraction.

5.2. NOS1 and cardioprotection

It has been consistently documented that NOS1 exerts a protective role in the myocardium in several model of stress, such as myocardial infarction [92, 93, 95], pressure overload [94, 102], ischemia-reperfusion [103], and dystrophic cardiomyopathy [104], just to mention the most relevant. Mechanistically, NOS1 nitrosylates several proteins, including ion channels [27, 29, 105] and mitochondrial components [106], and reduces oxidative stress [107].

A key hallmark of diabetic cardiomyopathy is hypertrophy. In this process, NO may play a role; furthermore, it has been shown that eNOS uncoupling (rather than the absence of NO) induces hypertrophy in the model of pressure overload [108]. Recoupling eNOS with BH_4

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reverses this process, but not the use of tempol, a ROS scavenger [109, 110]. In ob/ob mice, myocardial hypertrophy has been documented [111], along with reduced NO bioavailability associated with reduced levels of NOS1 and increased NADPH [34] and XOR activity [32].

The activity of NOS1 is required for basal phospholamban phosphorylation in the mouse heart [88, 89], with NOS1 uncoupling resulting in an impaired ventricular relaxation and diastolic dysfunction, as observed in a model of hypertension [112]. Conversely, an increased production of BH_4 by cardiac-specific overexpression of GTP cyclohydrolase 1 (GCH1, the rate-limiting enzyme for BH4 biosynthesis), accelerates myocardial relaxation [113]. NOS1 uncoupling in mdx cardiomyocytes is probably the result of the NOX2-derived oxidative stress, which ultimately leads to tetrahydrobioterin oxidation. With this in mind, one possibility is that NOS1 is found in a monomeric form resulting in uncoupling. This uncoupling has been proposed as the aftermath of excessive tetrahydrobioterin (BH_4), oxidation to dihydrobioterin (BH_2).

5.3. NOS2

Inducible nitric oxide synthase (iNOS or NOS2) appears to play a central role in the pathophysiology of the diabetic cardiomyopathy [54]. In the heart, like in most tissues, iNOS is not constitutively expressed. Inflammatory conditions trigger the activation of the nuclear factor kappa beta (NF-κB). The activity of iNOS has been usually ascribed as part of the nitrosative stress. Since iNOS activity is much higher than the constitutive isoforms (NOS1 and 3), it reacts with superoxide, producing peroxynitrite [83]. This oxidant reacts with the aromatic ring of tyrosine residues, generating nitrotyrosine, one of the most widespread markers of nitrosative and oxidative stress [114]. iNOS-derived NO depresses cardiac contractility and function [115], and has been described to be upregulated in patients with diabetic cardiomyopathy [116].

iNOS expression has been manipulated pharmacologically in diabetes, using resveratrol, a natural polyphenol present in red wine and grapes. Reveratrol reduces the oxidative stress, improves myocardial relaxation [117] and reduces iNOS expression in type-2 diabetes models, by reducing the activation of NF-κB [118].

5.4. NOS uncoupling

In physiological conditions, NOS catalyze the reaction of oxidation of L-arginine to produce NO and L-citrulline, in the presence of NADPH, oxygen, and the cofactor 6R-5,6,7,8-tetrahydrobioterin (BH_4). In the absence of BH_4, electrons coming from NADPH flow to molecular oxygen, but uncoupled of L-arginine, producing superoxide instead of NO [26]. Apparently, BH_4 stabilizes the NOS configuration as a dimer that favors the proper flow of electrons to L-arginine. In its absence, NOS monomerizes and only maintains its oxidase activity [119]. BH_4 and BH_2 have been shown to bind to NOS with similar affinity (Kd ~80 nM). Tetrahydrobiopterin essential cofactor for aromatic amino acid hydroxylases and NOS. BH_4 can be synthesized by two pathways: *de novo* or recycled from its oxidized forms (**Figure 3**). For *de novo* synthesis, GTP cyclohydrolase I (GTPCH), 6-pyruvoyl tetrahydrobioterin synthase (PTPS), and sepiapterin reductase (SR) convert GTP to BH_4. In the first reaction catalyzed by GTPCH, guanosine

5′triphosphate (GTP) is reduced to 7,8-dihydroneopterin 3′triphosphate (DNTP), being the rate-limiting reaction. Next, the conversion of DNTP to 6-pyrovoyl tetrahydrobioterin (PTP) is catalyzed by PTPS. The final reactions are two successive propyl side-chain reductions catalyzed by SR [39, 120].

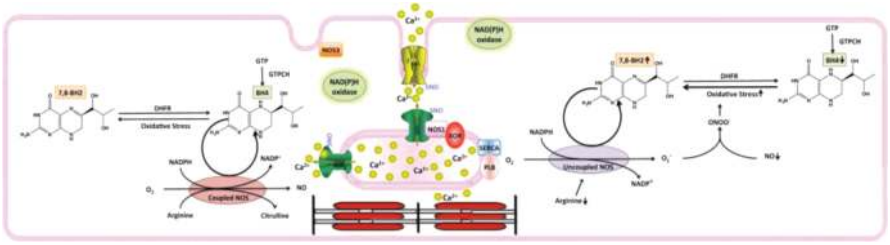

Figure 3. NOS uncoupling in the cardiomyocyte. Upon increased oxidative stress, BH_4, which normally couples L-arginine and NADPH oxidation and NO production, oxidized to BH_2, which is unable to couple this reaction, with the NADPH electrons passing directly to oxygen, producing superoxide.

When BH_4 is oxidized to BH_2, is reduced back to BH_4 by the enzyme dihydrofolate reductase (DHFR), in the salvage pathway. It is worth mentioning that during NOS catalysis BH_4 is not consumed, indicating that BH_2 formation is probably the result of other oxidative processes such as oxidative stress [39, 120].

5.5. NOS uncoupling in diabetes

It has been reported that NOS uncoupling takes places in diabetes, particularly in the vasculature [121]. For this reason, it has been associated with NOS3 [36, 37], the NOS isoform that is dominant in the endothelium. Given that oxidative stress is increased and that cardiac relaxation is impaired in diabetes, it is reasonable to think that NOS1 is uncoupled in the diabetic cardiomyocyte, since this isoform is related to phospholamban phosphoryaltion. Interestingly, it has been reported in the gastrointestinal system that diabetes produces NOS1 uncoupling, which is restored upon treatment with BH_4 [122] and sepiapterin, a BH_4 precursor [123].

The first report relating NOS uncoupling and diabetes was performed in cardiomyocytes exposed to high glucose concentration [124]. In these conditions, treatment with BH_4 restored the mechanic parameters in cardiomyocytes toward normal, an effect that could not be obtained using tetrahydroneopterin, which also shares ROS-scavenging properties as BH_4. Furthermore, the treatment of normal myocytes with the GTP cyclohydrolase I inhibitor 2,4-diamino-6-hydroxy-pyrimidine (DAHP) resembled the features of myocytes under hyperglycemic conditions.

The same group described that treatment of insulin-resistant mice with the BH_4 precursor folic acid was able to produce NOS recoupling. These effects were associated with improved cardiac function and reduction of sarcoplasmic reticulum calcium leak in isolated cardiomyocytes [125].

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Furthermore, Jo *et al*. described that treatment of streptozotocin-induced diabetic mice with the BH_4 precursor sepiapterin was able to restore the intracardiac BH_4 levels and restored the BH_4/BH_2 ratio in diabetic mice. This treatment recoupled NOS in wild type, eNOS- and nNOS-deficient mice, but not in iNOS-deficient mice, suggesting that iNOS uncoupling was responsible for the oxidative stress observed in type-1 diabetic mice. Importantly, this treatment restored ventricular function except in iNOS mice [126]. Apparently, NOS uncoupling is related to the increased activity of NADPH oxidase in streptozotocin-induced diabetic mice [55]. Furthermore, a recent report describes that in db/db mice, a model of type-2 diabetes, the coadministration of sepiapterin and L-citrulline,a precursor of L-arginine restored cardiac function in diabetic hearts and improved their response after ischemia-reperfusion [127].

6. Conclusion

In the diabetic heart, an increased hyperglycemia induced the mitochondrial production of ROS, which favors apoptosis. Concomitantly, diabetes induces the activation of NOX that further impairs mitochondrial function. In addition, the diabetic state induces the expression of iNOS, which in conjunction with an increased ROS production leads to the generation of peroxynitrite, a highly oxidizing species. These elements create a feed-forward system that deteriorates cardiac function at several levels. Furthermore, increased ROS production may oxidize BH4, producing NOS uncoupling, further aggravating the oxidative damage.

Despite the heavy burden that diabetes imposes to the heart, current therapeutic strategies do not specifically address diabetes-induced heart failure; therefore, novel pharmacological treatments might be of high relevance. Theoretically, a reduction in mitochondrial or NOX derived-oxidative stress in diabetic cardiomyopathy should be able to improve some of the features of diabetic cardiomyopathy. Nevertheless, indiscriminate NOX inhibition or ROS quenching may have detrimental effects if the protective role of NOX4 (as opposed as other NOXs) is disturbed, as it has been suggested in several stress conditions for the myocardium. On the other hand, inhibitors of mitochondrial ROS production are still in their infancy and have not been tested in humans. Therefore, pharmacological restoration of intracardiac BH_4 levels may offer a new therapeutic opportunity for diabetic cardiomyopathy, as they have been used in other pathologies, although the results have been somehow disappointing [128]. In addition, isoform-specific NOX inhibitors are needed as pharmacological tools against diabetic cardiomyopathy.

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