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REVIEW ARTICLE

New aspects of p66Shc in ischaemia reperfusion injury and other cardiovascular diseases

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Although reactive oxygen species (ROS) act as crucial factors in the onset and progression of a wide array of diseases, they are also involved in numerous signalling pathways related to cell metabolism, growth and survival. ROS are produced at various cellular sites, and it is generally agreed that mitochondria generate the largest amount, especially those in cardiomyocytes. However, the identification of the most relevant sites within mitochondria, the interaction among the various sources, and the events responsible for the increase in ROS formation under pathological conditions are still highly debated, and far from being clarified. Here, we review the information linking the adaptor protein p66Shc with cardiac injury induced by ischaemia and reperfusion (I/R), including the contribution of risk factors, such as metabolic syndrome and ageing. In response to several stimuli, p66Shc migrates into mitochondria where it catalyses electron transfer from cytochrome c to oxygen resulting in hydrogen peroxide formation. Deletion of p66Shc has been shown to reduce I/R injury as well as vascular abnormalities associated with diabetes and ageing. However, p66Shc-induced ROS formation is also involved in insulin signalling and might contribute to self-endogenous defenses against mild I/R injury. In addition to its role in physiological and pathological conditions, we discuss compounds and conditions that can modulate the expression and activity of p66Shc.

LINKED ARTICLES

This article is part of a themed section on Redox Biology and Oxidative Stress in Health and Disease. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v174.12/issuetoc

Abbreviations

ATG, DNA codon for methionine; CAD, coronary artery disease; CH, collagen homologues; $\Delta\psi_m$, mitochondrial membrane potential; ETC, electron transport chain; GSH/GSSG, GSH in its reduced or oxidized form; IGF-1, insulin-like growth factor 1; PTP, permeability transition pore; PBM, peripheral blood monocyte; Ras, rat sarcoma; SH2, sarcoma homologous type 2; Sirtuin, silent mating type information regulation 2 homolog

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Tables of Links

TARGETS	
Other protein targets[a]	**Enzymes**[c]
Bcl-2	Akt (PKB)
GPCRs[b]	Arginase II
α_1-adrenoceptor	ATP synthase
PAR1	Caspase-3
	Endothelial NOS
	ERK1
	ERK2
	JNK1
	MMP2
	mTOR
	PKCβ
	PTEN
	Sirtuin 1

LIGANDS	
β-amyloid	L-NAME
β-catenin	Nicotine
Acetylcholine	Nitric oxide (NO)
Angiotensin II	Oestradiol
Ascorbic acid	Palmitate
ATP	Propofol
Ethanol	Resveratrol
Glutathione (GSH)	Rottlerin
H_2O_2	Sotrastaurin
Homocysteine	Thrombin
IGF-1	Trichostatin
Insulin	Wnt-3a

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c]Alexander *et al.*, 2015a,b,c).

Cardiac injury induced by ischaemia and reperfusion

The maintenance of cardiac structure and function depends on the continuous supply of ATP resulting from the mitochondrial coupling of substrate oxidation with ATP synthesis, termed as oxidative phosphorylation. The strict dependence of the heart on aerobic metabolism is indicated by the large fraction (i.e. >30%) of cardiomyocyte volume occupied by mitochondria. Therefore, it is hardly surprising that mitochondrial dysfunction and cardiac diseases are inevitably associated. This concept is perfectly exemplified by cardiac injury induced by ischaemia and reperfusion.

Because more than 95% of oxygen is utilized by the terminal reaction of the respiratory chain, namely, cytochrome oxidase, anoxia or ischaemia is established when oxygen availability is no longer sufficient for the activity of cytochrome oxidase. Therefore, within any cell, ischaemic injury is determined primarily by mitochondrial dysfunction.

The arrest of electron flow causes inevitably a drop in mitochondrial membrane potential ($\Delta\psi_m$) that is the driving force for ATP synthesis by F_o–F_1 ATP synthase. Not only is ATP synthesis curtailed, but also its hydrolysis is stimulated to maintain $\Delta\psi_m$. To this aim, the F_o–F_1 ATP synthase inverts its physiological operation, becoming an ATPase that pumps protons from the matrix into the intermembrane space at the expense of ATP hydrolysis. In this way, mitochondria change from ATP producers into avid utilizers that hydrolyze not only intramitochondrial ATP but also cytosolic ATP produced by anaerobic glycolysis. These biochemical changes have profound functional consequences. On the one hand, the stimulation of glycolysis resulting in lactate formation causes intracellular acidification that is responsible for the asystole occurring in the first minutes of ischaemia. On the other hand, the drop in ATP content causes rigour contracture (Di Lisa *et al.*, 1998).

If coronary flow is re-established after a short ischaemic episode, viability is maintained, and contractility slowly recovers (Bolli and Marbán, 1999). However, when reperfusion occurs after a prolonged ischaemia (i.e. more than 20 min of no flow ischaemia in crystalloid-perfused isolated hearts), viability and contractility are no longer restored (Jennings *et al.*, 1975; Di Lisa *et al.*, 1998; Yellon and Hausenloy, 2007). In fact, under these conditions reperfusion seems to cause additional injury, because cardioprotection can be obtained by means of intervention applied at the onset of reperfusion (Ovize *et al.*, 2010).

Mitochondrial dysfunction is likely to be pivotal in determining the very rapid transition towards irreversible injury occurring upon reperfusion. In this respect, a seminal observation was the presence of calcium precipitates within swollen mitochondria in irreversibly-injured cardiomyocytes (Shen and Jennings, 1972). This finding implies that the readmission of oxygen allows mitochondria to recover $\Delta\psi_m$ required for mitochondrial Ca^{2+} uptake. In this process, mitochondria remove the excess Ca^{2+} in the cytosol that might activate proteases, phospholipases and signalling pathways jeopardizing cell survival. However, a large increase in Ca^{2+} uptake hampers ATP synthesis, because both these processes depend on $\Delta\psi_m$ (Bernardi, 1999). The resulting lack of recovery of ATP content in the presence of an elevated

intracellular [Ca^{2+}] is likely to promote hypercontracture eventually leading to sarcolemma rupture (Altschuld *et al.*, 1985; Siegmund *et al.*, 1991; Silverman and Stern, 1994). The dependence of this deleterious sequence of events on mitochondrial function is indicated by the attenuation of reperfusion-induced cell death induced by respiratory chain inhibitors or uncouplers of oxidative phosphorylation (Ganote *et al.*, 1976; Elz and Nayler, 1988). This notion indicates that during reperfusion, mitochondria are paradoxically essential for both functional recovery after a short ischaemic episode and cell death after a prolonged ischaemia.

Besides decreasing ATP synthesis, a large elevation in matrix [Ca^{2+}] further contributes to ATP depletion and mitochondrial de-energization by promoting the opening of the mitochondrial permeability transition pore (PTP) (Bernardi and Di Lisa, 2015). The elevation of intramitochondrial [Ca^{2+}] *per se* might be not sufficient to trigger PTP opening. In fact, in isolated mitochondria, PTP opening is obtained in the presence of [Ca^{2+}] >0.1 M, which is hardly attained in viable cells. Therefore, within intact cells, the PTP sensitivity to Ca^{2+} is likely to be increased by additional factors or processes among which ROS appear extremely relevant (Bernardi and Di Lisa, 2015). Post-ischaemic reperfusion causes a rapid elevation in ROS levels (Zweier, 1988; Bolli and Marbán, 1999) that has been recently demonstrated to occur within mitochondria *in vivo* (Chouchani *et al.*, 2013). ROS favours PTP opening that increases ROS formation generating a vicious cycle. This process termed as ROS-induced ROS release is likely to exacerbate the injury within a cardiomyocyte and spread it to adjacent cardiomyocytes (Zorov *et al.*, 2014).

Although the relevance of mitochondria in ROS formation is well established, especially under pathological conditions, it is far from clear which mitochondrial enzymes provide the largest contribution to oxidative stress.

Mitochondrial ROS formation

Mitochondria contain enzymes that catalyse hydrogen peroxide (H_2O_2) generation as the essential product along with systems generating ROS in sporadic, possibly undesired, reactions. This is especially the case with the electron transport chain (ETC) (Murphy, 2009; Chen and Zweier, 2014; Zorov *et al.*, 2014). Indeed, a minor fraction (about 0.1%) of the electrons flowing through the ETC is thought to cause the partial reduction of oxygen (O_2) into superoxide that is then rapidly reduced to H_2O_2 by superoxide dismutases (SODs) (Murphy, 2009). Therefore, H_2O_2 is the main ROS produced. The removal of H_2O_2 by mitochondrially targeted catalase has been shown to be cardioprotective (Schriner *et al.*, 2005; Anderson *et al.*, 2009; Dai *et al.*, 2011). Besides superoxide dismutation, peroxide handling is carried out by a thiol redox system centred on glutathione (GSH and GSSG in its reduced and oxidized form, respectively) and thioredoxin (Berndt *et al.*, 2007; Murphy, 2012; Forman *et al.*, 2014; Nickel *et al.*, 2014).

The physiological role of mitochondrial ROS generation in a wide variety of cardiomyocyte functions is likely to depend on post-translational modifications of proteins, especially at the level of cysteine residues (Finkel, 2012). Long-lasting changes are obtained by the effects of mitochondrial ROS on transcriptional factors, such as hypoxia-inducible factor (HIF) and nuclear factor erythroid 2-related factor 2 (Nrf2) (Hayes and Dinkova-Kostova, 2014; Semenza, 2014; Yun and Finkel, 2014). The detrimental effects on proteins, lipids, carbohydrates and nucleotides obtained in the presence of high levels of ROS explain the large body of evidence linking a high mitochondrial formation of ROS with every cardiac disease. This is especially the case with ischaemia–reperfusion injury.

Besides respiratory chain complexes, several other mitochondrial enzymes, such as glycerol-3-phosphate and 2-oxoglutarate dehydrogenase, have been described as potential ROS producers. The list of dedicated enzymes for ROS formation in mitochondria might include nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4; Ago *et al.*, 2010), although results from a recent study argued against its mitochondrial localization in the normoxic heart (Hirschhäuser *et al.*, 2015).

However, the contribution of the respiratory chain and mitochondrial dehydrogenases to the overall ROS production of mitochondria *in vivo* cannot be defined precisely, because their deletion would inevitably compromise functions other than ROS generation. The demonstration that mitochondria generate ROS *in vivo* is provided by studies on other mitochondrial enzymes, such as p66Shc and monoamine oxidases that generate H_2O_2 as a direct and obligatory product. While the relevance of monoamine oxidase (MAO) in cardiac diseases has been covered in a recent review (Kaludercic *et al.*, 2014), the role of p66Shc in cardiovascular pathophysiology is detailed in the following sections.

p66Shc structure, function and expression

Biochemical features

p66Shc is a ubiquitously expressed vertebrate protein. It sustains the intracellular concentration of ROS by catalysing their formation from the mitochondrial respiratory chain, by triggering plasma membrane oxidases and by suppressing ROS scavenging (see for review Trinei *et al.*, 2009; Trinei *et al.*, 2013).

P66Shc is one of the three isoforms encoded by the human and mouse ShcA locus. ShcA was identified in 1992 by low-stringency hybridization to human cDNA libraries, using a sarcoma homologous type 2 (SH2) coding sequence as a probe (Pelicci *et al.*, 1992). It is located on chromosome 1q21. The originally isolated ShcA transcript displayed two in-frame ATGs and was shown to encode two ubiquitously expressed polypeptides: p52Shc and p46Shc.

Members of the spontaneous human combustion (Shc) family are characterized by the phosphotyrosine binding domain (PTB) –collagen homologues 1 (CH1)–SH2 modularity where CH1 is a glycine/proline rich region, containing two major phosphorylation sites (tyrosine 239–240 and tyrosine 317), SH2 is the C-terminal domain involved in protein–protein interaction and the formation of multi-protein signalling complexes and PTB is able to bind

non-phosphorylated tyrosine-containing peptides. p66Shc contains an additional amino-terminal proline rich region, named CH2.

Relations with signalling pathways and H_2O_2 production

Isoforms p46Shc and p52Shc serve as phosphotyrosine adaptor molecules in various receptor-mediated signalling pathways. Once phosphorylated, they recruit the growth factor receptor-bound protein 2 (Grb2)/son of sevenless (SOS) complex to the plasma membrane (Ravichandran, 2001) and subsequently activate the MAPK cascade. p52Shc and p46Shc function as initiators of the rat sarcoma (Ras) signalling cascade in various non-neuronal systems (Migliaccio *et al.*, 1997). p66Shc is a target of receptor tyrosine kinases (Okada *et al.*, 1997) and is able to bind the Grb2/SOS complex. However, p66Shc is not involved in Ras signalling regulation, and its overexpression has a negative effect on the Ras–MAPK–fos pathway in response to EGF. On the contrary, p66Shc converts stress signals into apoptosis (Migliaccio *et al.*, 1999).

A fraction of p66Shc has been observed within the mitochondrial intermembrane space, although the import mechanism of p66Shc into mitochondrial intermembrane space has not been clearly identified. During apoptosis, p66Shc levels in mitochondria increase. Some stress kinases such as JNK-1 and PKCß phosphorylate p66Shc on serine 36, and peptidyl-prolyl *cis–trans* isomerase-1 induces its prolyl-isomerization (Pinton *et al.*, 2007); these post-translational modifications are involved in p66Shc translocation into mitochondria. In basal conditions, mitochondrial p66Shc associates to a high molecular weight complex of about 670 kDa and to heat shock protein 70 (Orsini *et al.*, 2006) and components of the transporter of the outer and inner mitochondrial membrane complex (Cosentino *et al.*, 2008). Notably, treatment of cells with pro-apoptotic stimuli such as ultraviolet light or H_2O_2 induces the dissociation of these complexes and the release of monomeric p66Shc, which reacts with cytochrome c (Orsini *et al.*, 2006) sequestering electrons from the ETC to generate H_2O_2 (Giorgio *et al.*, 2005; Trinei *et al.*, 2013). Through this process, p66Shc appears to provide an important contribution to mitochondrial ROS formation that has been highlighted in a wide array of physiological and pathological conditions (Figure 1).

Modulation of p66Shc expression in the heart

Compared with wild-type littermates, p66Shc knockout mice had similar blood pressure, heart rate and left ventricular wall thickness. However, cardiomyocyte number was increased in p66Shc knockout mice (Graiani *et al.*, 2005). One potential explanation for a decreased cardiomyocyte number at a similar myocardial wall thickness and cardiac weight (implying hypertrophied cardiomyocytes) in wild-type mice relates to the expression of p66Shc that was detected only in neonatal, but not in adult cardiomyocytes (Obreztchikova *et al.*, 2006). In cardiomyocyte cultures from 2-day-old rats, an α_1-adrenoceptor ROS-dependent p66Shc–Akt–forkhead box O3 (FOXO3) phosphorylation pathway decreased SOD2 expression, thereby impairing ROS detoxification and increasing cardiomyocyte apoptosis. Furthermore, in these cardiomyocytes, p66Shc acted as a negative regulator of

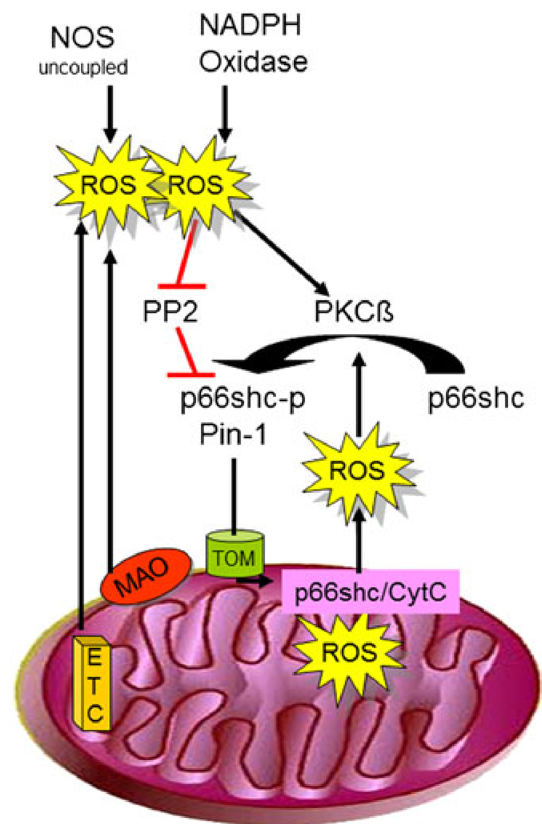

Figure 1

ROS derived from uncoupled NOS, NAPDH oxidase, monoamine oxidases (MAO) or the ETC activate PKCß and – at the same time – inhibit protein phosphate 2 (PP2). Both PKCß activation and PP2 inhibition contribute to increased p66Shc phosphorylation, which then translocate in the presence of peptidyl-propyl *cis–trans* isomerase 1 (Pin-1) into the mitochondrial intermembrane space using the translocator of the outer mitochondrial membrane (TOM). p66Shc then catalyses electron transfer from cytochrome c to oxygen, resulting in increased ROS formation.

hypertrophy (Guo *et al.*, 2009), potentially explaining the above differences found in *in vivo* hearts.

Ageing, comparing 6- and 36-month-old Fischer 344xBN rats, was associated with decrements in cardiac mitochondrial content and respiratory function. Mitochondria from aged hearts demonstrated a greater enrichment of p66Shc (Ljubicic *et al.*, 2010). Similarly, intense exercise activated p66Shc; in male Wistar rats, swimming for 3 h per day increased p66Shc phosphorylation, surprisingly without a significant increase in either mitochondrial ROS release or mitochondrial oxidative stress markers or the antioxidant enzyme activities (SOD and catalase) (Ziolkowski *et al.*, 2015). Thus, regular exercise and ageing affect cardiac p66Shc expression and/or activity; however, the question whether such an increase in p66Shc expression and/or activity relates to cardiomyocyte mitochondria or to mitochondria obtained from other cell types (such as fibroblasts) cannot be resolved using whole cardiac homogenates and remains to be answered using isolated cell preparations.

p66Shc expression appears to be low in cardiomyocytes under physiological conditions, yet it can be induced by various

stimuli. *In vitro* experiments showed that angiotensin II caused apoptotic death of cardiomyocytes isolated from wild-type but not p66Shc knockout mice hearts (Graiani *et al.*, 2005). Consistent with the *in vitro* results, infusion of a subpressor dose of angiotensin II (300 nmol·kg^{-1} body weight daily for 28 days) caused left ventricular hypertrophy and apoptotic death of cardiomyocytes and endothelial cells in wild-type but not p66Shc knockout mice (Graiani *et al.*, 2005).

Thrombin activates protease-activated receptor-1 (PAR1) and engages signalling pathways that influence the growth and survival of cardiomyocytes as well as extracellular matrix remodelling by cardiac fibroblasts. Thrombin increased p66Shc phosphorylation at serine 36 in cardiac fibroblasts and cardiomyocytes, the latter requiring increased PKC and MAPK/ERK activities. *Pasteurella multocida* toxin, a Gαq agonist, also promoted p66Shc expression and cardiomyocyte hypertrophy (Obreztchikova *et al.*, 2006).

p66Shc and cardiac pathologies

Ischaemia/reperfusion injury

Initial evidence for p66Shc involvement in ischaemia/reperfusion injury was obtained by showing that in hindlimb ischaemia, p66Shc deletion prevented the decrease in both capillary density and tissue viability (Zaccagnini *et al.*, 2004). p66Shc deletion in satellite muscle and endothelial cells protected against apoptosis by reducing ROS formation. Cardiac protection was demonstrated in isolated mouse hearts undergoing ischaemia and reperfusion. p66Shc deletion resulted in a decrease in both lactate dehydrogenase release reflecting viability maintenance and parameters of oxidative stress, such as malondialdehyde formation and tropomyosin oxidation (Carpi *et al.*, 2009). Interestingly, this cardioprotective efficacy associated with p66Shc ablation was comparable with that of other antioxidant interventions and could not be increased by antioxidant co-administration, thus suggesting that p66Shc is downstream of other pathways involved in ROS formation.

In isolated guinea pig hearts, phosphorylation of p66Shc and its translocation into mitochondria increased during reperfusion after 20 and 30 min ischaemia, but not during ischaemia only, or during 5 or 10 min ischaemia followed by 20 min reperfusion. Amobarbital, a complex I blocker, or hispidin, a PKCß inhibitor, reduced p66Shc phosphorylation and its mitochondrial translocation induced by 30 min ischaemia and 20 min reperfusion. Decreased phosphorylation of p66Shc by amobarbital or hispidin led to better functional recovery and less infarction during reperfusion (Yang *et al.*, 2014).

In contrast to the above finding, when reperfusion was established after 30 min of ischaemia, p66Shc knockout was associated with larger infarcts compared with wild-type mice. p66Shc inhibition was not associated with modifications in post-infarction inflammation, oxidative burst, cardiac vessel density or structure. Thus, genetic deletion of p66Shc increased susceptibility to myocardial injury in response to reperfusion after short-term ischaemia (Akhmedov *et al.*, 2015). Notably, in the *in vivo* mice study, infarct size following 30 ischaemia reperfusion after 30 min of ischaemia was associated with an infarct size much lower than that commonly detected in other laboratories. Furthermore, the difference in infarct size between wild-type and p66Shc knockout mice was minimal, if not negligible, casting doubts on its biological/clinical importance. Finally, p66Shc phosphorylation or translocation to mitochondria in wild-type mice was not assessed.

Besides significant technical concerns of the above study, there is indeed the potential of p66Shc being protective during short periods of ischaemia. p66Shc might increase the resistance to shorter periods of ischaemia by activating Akt (as part of the so-called reperfusion injury salvage kinase pathway) through increased phosphorylation. Akt phosphorylation can be enhanced by inhibition of protein phosphatases, among which protein tyrosine phosphatases are inactivated by ROS via oxidation of critical cysteinyl residues. Indeed in fibroblasts, p66Shc deletion decreased oxidation and thus inactivation of protein phosphatases associated with a reduced activation of ERK and Akt (Frijhoff *et al.*, 2014).

In p66Shc knockout mice, activation of the protective and anti-apoptotic reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement pathways following 30 min ischaemia and reperfusion were blunted, and mitochondrial swelling and cellular apoptosis via the caspase-3 pathway increased compared with wild type mice (Akhmedov *et al.*, 2015).

Therefore, p66Shc-induced ROS formation could contribute to endogenous self-defenses triggered by a short/mild ischaemic episode, yet no information is available on the role of p66Shc in the protection attributed to conditioning stimuli. However, p66Shc-induced ROS formation would become detrimental when exacerbated upon reperfusion after a long/severe ischaemia or prolonged over time as appears to be the case with hypertensive or diabetic cardiomyopathy (Figure 2).

Surprisingly, little attention has been given to the role of p66Shc in post-myocardial infarction changes. Recently, evidence has been provided that p66Shc deletion improves myocardial healing and reduces cardiac fibrosis (Baysa *et al.*, 2015). In particular, the absence of p66Shc was associated with a striking decrease in the incidence of cardiac rupture. The obvious elimination of the ruptured hearts from functional evaluation might explain the lack of a significant difference in

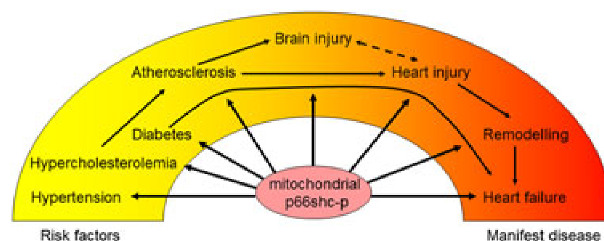

Figure 2

Increased translocation of p66Shc into mitochondria results in increased ROS formation. Indeed, p66Shc activation contributes to several cardiovascular pathologies including diabetes, atherosclerosis, acute brain and heart injury as well as cardiac remodelling and failure. Thus, p66Shc may be an interesting target to attenuate cardiovascular disease progression.

contractile function between post-myocardial infarction hearts from p66Shc knockout mice and wild-type littermates. The study of the underlying mechanism highlighted fibroblast activation along with increased collagen formation and reduced activation of MMP2 in p66Shc knockout hearts. These findings are likely to explain the protection against adverse remodelling in p66Shc knockout hearts. Interestingly, in isolated fibroblasts, p66Shc deletion was associated with a decrease in MMP2 expression, while an increase was observed when p66Shc was overexpressed. Nevertheless, regulation of oxidative activation of MMP2 by p66Shc may suggest its involvement in ischaemia/reperfusion injury and endogenous cardioprotection (Lalu *et al.*, 2002).

Myocardial hypertrophy and failure

The observation that p66Shc expression and/or activity was induced by angiotensin II, thrombin and a Gα(q) agonist leading to cardiomyocyte hypertrophy and apoptosis (see Modulation of p66Shc expression in the heart section) suggests the involvement of p66Shc in cardiac hypertrophy and failure.

Indeed, alcohol- and diabetes-induced cardiomyopathy has been linked to p66Shc. High-dose alcohol induced apoptosis in cardiomyocytes, which was associated with a dose-dependent phosphorylation of p66Shc and ROS formation. Exposure to alcohol also led to the loss of $\Delta\psi_m$ and cytochrome c release. Depletion of p66Shc and inhibition of PKCß successfully reversed these effects and suppressed the alcohol-induced apoptosis in cardiomyocytes (Wang *et al.*, 2015). While alcohol appeared to act directly on cardiomyocytes, in a model of insulin-dependent diabetes mellitus, the generation of ROS led to telomeric shortening and apoptosis of cardiac progenitor cells, thereby impairing the growth reserve of the heart. Ablation of the p66Shc gene prevented these negative adaptations of the cardiac progenitor cell compartment, interfering with the development of heart failure in diabetes (Rota *et al.*, 2006). Through p66Shc and sirtuin 1, diabetes and sympathectomy elevated the expression of various adhesion molecules on stem and progenitor cells (including CD62 antigen-like family member L), thereby inhibiting their mobilization. Knockout of CD62 antigen-like family member L (CD62L) partially restored the defective stem/progenitor cell mobilization (Albiero *et al.*, 2014). More general information on the relationship between p66Shc and diabetes is provided in the P66Shc and cerebral pathologies section.

Taken together, these finding indicate that there is no doubt that with prolonged stresses, p66Shc contributes to cardiac pathologies, such as the development of heart failure. Regarding cardiac ischaemia/reperfusion injury, p66Shc might be either be protective or deleterious depending upon the duration and severity of ischaemia. At present, data are sparse, and no evidence exists whether or not p66Shc is involved in endogenous cardioprotection.

P66Shc and vascular abnormalities

Endothelial dysfunction and cardiovascular risk factors

Increased expression of p66Shc was reported to be associated with several stimuli or conditions leading to endothelial dysfunction, while p66Shc deletion resulted in beneficial effects. Major examples are as follows.

- *Angiotensin II and pressure overload.* Aortic segments from mice exposed to increased blood pressure secondary to transaortic constriction showed a decreased phosphorylation of endothelial NOS, an increased p66Shc phosphorylation and superoxide production (Lee *et al.*, 2008). The increase in p66Shc expression was most likely a consequence of the activation of the renin–angiotensin system secondary to transaortic constriction, because angiotensin II *per se* elicited an increase in p66Shc phosphorylation in cultured mice endothelial cells (Lee *et al.*, 2008).
- *Hypercholesterolaemia* up-regulated human endothelial cell p66Shc expression leading to a dysfunctional endothelial cell surface with pro-adhesive and procoagulant features (Kim *et al.*, 2012). In mice *in vivo*, high-fat diet induced endothelial dysfunction that was associated with increased endothelial Wnt3a, dephosphorylated β-catenin and phosphorylated p66Shc expression. High-fat diet-induced dephosphorylation of endothelial β-catenin was diminished in p66Shc knockout mice (Vikram *et al.*, 2014). Hypercholesterolaemia also led to a prothrombotic phenotype on platelets. In wild-type mice on high-fat diet, surface P-selectin expression on platelets and platelet aggregation induced by thrombin were increased. These exaggerated platelet responses induced by high-fat diet were significantly blunted in p66Shc siRNA-treated mice (Kumar *et al.*, 2014).
- *Diabetes*. p66Shc-induced ROS formation is involved not only in the disorders caused by diabetes but also in its onset. This notion is hardly surprising, because p66Shc is critical in maintaining insulin-dependent signalling and glucose homeostasis in a variety of tissues (Tomilov *et al.*, 2011). In particular, in adipocytes, insulin induces the phosphorylation of p66Shc (serine 36). The consequent p66Shc-catalysed ROS production causes the oxidation of specific phosphatases, resulting in their inactivation. Major examples are given by phosphatase and tensin homolog (PTEN; Berniakovich *et al.*, 2008) and protein tyrosine phosphatase 1B (Frijhoff *et al.*, 2014) that regulate the intracellular insulin transduction cascade. Consequently, p66Shc potentiates insulin signalling and regulates insulin-induced gene expression and triglyceride accumulation in adipocytes (Berniakovich *et al.*, 2008).

Regarding its involvement in diabetes, p66Shc is activated by hyperglycaemia (Pagnin *et al.*, 2005), and the consequent H_2O_2 generated by p66Shc within mitochondria promotes the loss of viability in tissues of diabetic mice (Menini *et al.*, 2007; Fadini *et al.*, 2010; Cheng *et al.*, 2013) or patients (Albiero *et al.*, 2014).

Mechanistically, p66Shc appears to antagonize insulin and mammalian target of rapamycin (mTOR) signalling, which limits glucose uptake and metabolism (for review, see Soliman *et al.*, 2014).

The data available support a model in which adipose PKCß activation is among the initiating events that disrupt mitochondrial function through interaction with p66Shc and amplify fat accumulation and adipose dysfunction with systemic consequences. Manipulation of PKCß levels, activity or signalling could provide a therapeutic approach to combat

obesity and associated metabolic disorders (for review, see Mehta and Mehta, 2014, as well as Diogo *et al.*, 2013).

Remarkably, p66Shc knockout mice are protected from diet-induced obesity, the associated pro-inflammatory state and decrease in glucose tolerance (Berniakovich *et al.*, 2008), although they are more sensitive to cold stress and starvation (Giorgio *et al.*, 2012). Also, in lep$^{Ob/Ob}$ mice, an established model of obesity and insulin resistance, p66Shc deletion was found to improve glucose tolerance without affecting (hyper)insulinaemia and independently of body weight (Ranieri *et al.*, 2010). However, more recently in the same model, a decrease in body weight was not associated with changes in glucose tolerance, insulin resistance and adipose tissue remodelling (Ciciliot *et al.*, 2015).

In diabetic mice, up-regulation of p66Shc contributed to impaired vascular reactivity of the cavernosal tissue (Cheng *et al.*, 2013), and p66Shc was shown to be involved in the delayed skin wound healing process in the setting of diabetes and ischaemia (Fadini *et al.*, 2010).

In patients, body mass index was correlated with p66Shc protein levels derived from adipose tissue. An additional clinical report correlated the higher level of p66Shc mRNA in peripheral mononuclear blood cells from type 2 diabetic patients with elevated markers of oxidative stress in plasma (Pagnin *et al.*, 2005).

- *Oxidative stress and lipoproteins*. LDL cholesterol becomes modified by ROS, and oxidized LDL cholesterol binding to its receptor (lectin-like oxidized LDL receptor-1) further increases ROS formation. Incubation of human aortic endothelial cells with oxidized LDL cholesterol increased p66Shc phosphorylation at serine 36 (Shi *et al.*, 2011; Shi *et al.*, 2014a). Blockade of the lectin-like oxidized LDL receptor-1 prevented p66Shc phosphorylation as did inhibition of PKCßII and JNK. p66Shc silencing blunted oxidized LDL cholesterol induced ROS production (Shi *et al.*, 2011). Apurinic/apyrimidinic endonuclease 1 suppressed oxidized LDL cholesterol-induced p66Shc activation in endothelial cells by inhibiting PKCß-mediated p66Shc phosphorylation and subsequently prevented vasoconstriction induced by activation of PKC (Lee *et al.*, 2011).
- *NOS*. Further supporting the importance of p66Shc for endothelial cell (dys)function, overexpression of p66Shc inhibited endothelial NOS-dependent NO production (Yamamori *et al.*, 2005), in part by uncoupling of endothelial NOS (Shi *et al.*, 2014a). siRNA-mediated down-regulation of endogenous p66Shc activated Akt kinase and subsequently increased serine 1177 phosphorylation of endothelial NOS. In rat aortic rings, down-regulation of p66Shc suppressed the vasoconstrictor response to phenylephrine that was prevented by treatment with the NOS inhibitor L-NAME, and enhanced vasodilation induced by submaximal doses of acetylcholine (Yamamori *et al.*, 2005).
- Finally, ageing affects p66Shc expression and vascular function. Endothelium-dependent relaxation was age-dependently impaired in aortic rings (Francia *et al.*, 2004) and cerebral arteries, but not in femoral arteries of wild-type mice (Shi *et al.*, 2014b). This process was paralleled by an increase in ROS production and was mediated by the *p66Shc* gene since p66Shc knockout abolished the age-dependent decrease in endothelial function.

Thus, many of the known cardiovascular risk factors increase p66Shc expression and/or activity and subsequently impair endothelial cell function and vascular reactivity (for details, see Lizama-Manibusan and McLaughlin, 2013; Magenta *et al.*, 2014; Grimaldi *et al.*, 2015).

Plaque development

Subsequent to endothelial dysfunction, plaque development occurs leading to coronary artery disease (CAD). This well-established sequence of events appears to both involve p66Shc and be prevented by its deletion. Major lines of evidence are summarized as follows.

- *High-fat diet*. The atherosclerotic lesion area resulting from chronic high-fat diet was reported to increase more in wild-type than p66Shc knockout mice. Early lesions from p66Shc knockout mice had fewer macrophage-derived foam cells and apoptotic vascular cells in comparison with those from wild-type mice (Napoli *et al.*, 2003). Similar results were obtained in hypercholesterolaemic apolipoprotein E knockout mice in which p66Shc knockout attenuated atherosclerotic development caused by a high fat diet (Martin-Padura *et al.*, 2008).
- *IGF-1*. This endocrine and autocrine/paracrine growth factor circulates at high levels in the plasma and is expressed in most cell types (for review, see Higashi *et al.*, 2012). p66Shc inhibited IGF-I signalling, leading to attenuation of IGF-I-stimulated vascular smooth muscle cell proliferation and migration (Xi *et al.*, 2008).

In vascular smooth muscle cells overexpressing wild-type arginase II, mitochondrial dysfunction and cell apoptosis occurred, which were abrogated by p66Shc silencing. The activation of p66Shc by arginase II was dependent on ERK and sequential activation of 40S ribosomal protein S6 kinase 1 – JNKs (Xiong *et al.*, 2013).

Thus, deletion of p66Shc protected against endothelial dysfunction and atherosclerotic plaque formation in mice fed a high-fat diet (Martin-Padura *et al.*, 2008). Moreover, P66Shc overexpression has been shown to mediate platelet activation and aggregation in hypercholesterolaemia in both mouse and human platelets (Kumar *et al.*, 2014).

- *CAD*. The p66Shc mRNA and protein expression levels in peripheral blood leukocytes were significantly higher in CAD patients compared with control patients. Interestingly, the expression of p66Shc positively correlated with the serum homocysteine level. The mechanisms linking p66Shc and homocysteine are not yet elucidated. However, the homocysteine-p66Shc connection appears to involve CpG methylation in the p66Shc promoter. In fact, plasma homocysteine levels showed a significant difference between CAD patients with a high or low degree of CpG methylation in peripheral blood leukocytes. Thus, homocysteine appears to up-regulate human p66Shc expression via hypomethylation of specific CpG dinucleotides in the p66Shc promoter (Kim *et al.*, 2011).

Besides changes in promoter methylation, p66Shc RNA levels were increased in peripheral blood monocytes (PBMs)

of acute coronary syndrome patients as compared with stable CAD patients and controls. Furthermore, oxidative stress (malondialdehyde levels) increased in plasma of acute coronary syndrome patients, and levels of malondialdehyde correlated positively with p66Shc (Franzeck *et al.*, 2012). Thus, *p66Shc* gene expression level in PBMs may be viewed as a marker of CAD in humans (Noda *et al.*, 2010).

Flow-mediated dilatation was lower and the carotid intima-media thickness higher in coronary heart disease patients. Notably, in a multiple linear regression analysis, flow-mediated dilatation was inversely correlated with p66Shc mRNA expression, again pointing to a pivotal role for the expression of p66Shc in endothelial dysfunction (Miao *et al.*, 2015).

Taken together, these findings provide clear evidence that p66Shc contributes to vascular pathologies such as endothelial dysfunction or plaque formation and that silencing of p66Shc through improvement of the NO/ROS balance exerts protective effects.

P66Shc and cerebral pathologies

Ischaemia/reperfusion injury

A role for p66Shc in cardiac susceptibility to ischaemia/reperfusion injury and other pathological conditions is also relevant in neuronal cells.

In wild-type mice, post-ischaemic p66Shc knockdown preserved blood–brain barrier integrity resulting in improved stroke outcome, as identified by smaller lesion volumes, decreased neurological deficits and increased survival (Spescha *et al.*, 2015). Thus, p66Shc appears to be involved in irreversible brain injury following ischaemia/reperfusion. Similarly, p66Shc knockout protected against experimental autoimmune encephalomyelitis in mice without affecting the overall immune response; reduced neuronal cell death most likely occurred through the delayed opening of the PTP in p66Shc knockout mice (Savino *et al.*, 2013).

Similar to CAD, in stroke patients, p66Shc gene expression in PBM was transiently increased, and this increase correlated with short-term neurological outcome (Spescha *et al.*, 2015).

In an *in vitro* neuronal culture model, p66Shc activation occurred within 1 h of preconditioning ischaemia. Phosphorylated p66Shc rapidly relocalized to the mitochondria, and preconditioned cells experienced increased oxidative stress. Inhibiting p66Shc activation during preconditioning blocked this neuroprotection, suggesting that p66Shc was critical for preconditioning protection (Brown *et al.*, 2010). In human neuroblastoma cells, a brief preconditioning stress induced by serum deprivation for 2 h mediated tolerance against subsequent lethal oxidative stress. Preconditioning stress concomitantly up-regulated the expression of the anti-apoptotic B-cell lymphoma-2 (Bcl-2) protein and down-regulated the p66Shc adaptor protein during the lethal stress period (Andoh *et al.*, 2000). However, there is still a lack of *in vivo* brain conditioning studies.

Other pathologies

In mouse hippocampal HT22 cells, angiotensin II up-regulated the expression of PKCßII and induced p66Shc serine 36 phosphorylation and facilitated p66Shc mitochondrial translocation resulting in increased ROS formation, mitochondrial cytochrome c release, caspase-3 activation and the inhibition of cell viability. Interestingly, propofol inhibited the angiotensin II-induced PKCßII expression, p66Shc mitochondrial translocation, ROS formation, mitochondrial cytochrome c release, caspase-3 activation and finally improved cell viability (Zhu *et al.*, 2014).

In rat glioblastoma cells, β-amyloid-induced ROS production was observed in the presence of p66Shc leading to cell death. ROS scavengers and p66Shc knockdown decreased ROS formation and cell death (Bashir *et al.*, 2014).

Taken together, these data from *in vitro* and *in vivo* experiments indicate that p66Shc contributes to several brain pathologies but that it might also be involved in neuroprotection depending on the strength of the stimulus.

Therapeutic potential of p66Shc inhibition

Several exogenous factors including synthetic chemicals and plant compounds have been demonstrated to boost or suppress p66Shc gene expression or protein function (Table 1). Consequently, both the activation and inhibition of p66Shc activity might be pursued as therapeutic strategies. A reduced expression and/or activity should be imposed when the loss of tissue viability has to be prevented, such as in the case of ischaemic syndromes, while anticancer therapies are likely to be potentiated by enhancing p66Shc-induced ROS formation.

Activators

The activators of p66Shc belong to diverse chemical classes and share the ability to increase intracellular oxidative stress (Orsini *et al.*, 2006).

Different toxic chemicals that trigger cell death increase p66Shc levels or activate it by stimulating Ser^{36} phosphorylation including ethanol (Wang *et al.*, 2015), nicotine (Arany *et al.*, 2013a), anticancer taxanes (Arany *et al.*, 2013b) and the neurotoxin hydroxydopamine (Yamamori *et al.*, 2011). Notably, p66Shc levels do not always correlate with increased cell death. In fact, although in ovarian and prostate cancer cells, p66Shc is up-regulated by steroids as a result of decreased ubiquitination, the increase in p66Shc levels correlates with cell proliferation (Kumar *et al.*, 2011). Conversely, in non-transformed cells, oestradiol was reported to inhibit p66Shc translocation to mitochondria. This effect might explain the ability of oestradiol to protect against mitochondria-generated apoptosis in several non-reproductive tissues (La Colla *et al.*, 2015).

Among known activators of sirtuin 1, resveratrol was found to induce p66Shc expression and Ser^{36} phosphorylation in a process dependent on ERK1/2 activation (Fabbrocini *et al.*, 2010). Salvianolic acid A extracted from *Salvia miltiorrhiza*, another polyphenol that increases sirtuin 1, inhibited p66Shc transcription in hepatocytes (Xu *et al.*, 2013).

Inhibitors

The addition of palmitate increased the expression of p66Shc in different cell types (Favre *et al.*, 2015). However,

Table 1

p66Shc modulation by compounds and interventions of therapeutic interest

Compounds or stimuli	Overall effect	P66Shc expression	P66Shc Ser36 phosphorylation	P66Shc translocation to mitochondria	Oxidative changes of p66Shc	References
Ethanol	Activation		↑		↑	Wang *et al.*, 2015
Nicotine	Activation		↑		↑	Arany *et al.*, 2013a
Taxanes	Activation		↑		↑	Arany *et al.*, 2013b
Hydroxydopamine	Activation		↑		↑	Yamamori *et al.*, 2011
Steroids (in transformed cells)	Activation	↑				Kumar *et al.*, 2011
Steroids (in non-transformed cells)	Inhibition		↓			La Colla *et al.*, 2015
Caloric restriction and fasting	Inhibition	↓	↓			Giorgio *et al.*, 2012
Exercise	Inhibition	↓	↓			Santos-Alves *et al.*, 2014, 2015
Exposure to cold	Activation	↑				Giorgio *et al.*, 2012
High-fat diet	Activation	↑				Berniakovich *et al.*, 2008; Tomilov *et al.*, 2011
Palmitate	Activation	↑				Favre *et al.*, 2015
Polyunsaturated fatty acids	Inhibition		↓			Jing *et al.*, 2014
Resveratrol	Activation		↑			Fabbrocini *et al.*, 2010
Salvianolic acid A	Inhibition	↓				Xu *et al.*, 2013
PKC inhibitors	Inhibition		↓			Fuller *et al.*, 2012; Pinton *et al.*, 2007; Song *et al.*, 2014
Amobarbital	Inhibition			↓		Yang *et al.*, 2014
Hispidin	Inhibition			↓		Yang *et al.*, 2014
Sotrastaurin	Inhibition			↓		Fuller *et al.*, 2012
H_2S	Inhibition		↓		↑	Xie *et al.*, 2014
Trichostatin	Inhibition		↓			Kang *et al.*, 2015
Polysaccharide from *Ganoderma lucidum*	Inhibition	↓	↓			Kirmani *et al.*, 2013
Ascorbic acid	Inhibition	↓	↓			Kirmani *et al.*, 2013
SHetA2	Inhibition			↓		

polyunsaturated fatty acids that activate the AMP kinase/sirtuin 1 pathway suppressed p66Shc (serine36) phosphorylation (Jing *et al.*, 2014).

The PKC inhibitors hispidin (Pinton *et al.*, 2007), rottlerin (Song *et al.*, 2014) and sotrastaurin (Fuller *et al.*, 2012) were found to suppress p66Shc phosphorylation. Notably, amobarbital, hispidin (Yang *et al.*, 2014) or sotrastaurin (Fuller *et al.*, 2012) prevented the mitochondrial translocation of p66Shc occurring in cardiomyocytes upon ischaemia/reperfusion, reducing injury and improving recovery after myocardial infarction. More recently, an additional link with signalling pathways has been described relating p66Shc with hydrogen sulphide (H_2S) (Xie *et al.*, 2014). In particular, sulfhydration of Cys^{59} has been shown to disrupt p66Shc interaction with PKCβII, eventually hampering its phosphorylation at Ser^{36} and the consequent translocation into mitochondria. p66Shc sulfhydration is likely to play a relevant role in both the antioxidant property of H_2S and its cardioprotective effects (Andreadou *et al.*, 2015).

Trichostatin was also found to inhibit p66Shc phosphorylation, especially in the presence of angiotensin II (Kang *et al.*, 2015). Less clear is how the polysaccharide extract from *Ganoderma lucidum* or ascorbic acid attenuated the expression and phosphorylation of p66Shc (Kirmani *et al.*, 2013).

Finally, the only compound known to directly bind p66Shc, thus preventing its association with heat shock protein 70 and mitochondrial import, is the synthetic heteroarotinoid SHetA2 (Benbrook *et al.*, 2014). However, effects on tissue degeneration have not yet been reported.

In addition to chemical compounds, lifestyle interventions also affect p66Shc expression/activity. Caloric

restriction, fasting (Giorgio *et al.*, 2012) and exercise (Santos-Alves *et al.*, 2014, 2015) were reported to reduce p66Shc gene expression and p66Shc (serine 36) phosphorylation in mouse liver, fat and muscle. In contrast, cold exposure or obesogenic high fat diet increase p66Shc protein levels in mouse fat and muscle (Berniakovich *et al.*, 2008; Tomilov *et al.*, 2011; Giorgio *et al.*, 2012).

Some open questions relating to p66Shc

- Does p66Shc decrease or increase irreversible tissue injury following ischaemia/reperfusion?

In the heart, in particular, the data are controversial with p66Shc knockdown either decreasing or increasing infarct size following short periods of ischaemia/reperfusion (see above). While methodological and/or species differences might contribute to these divergent findings, p66Shc could indeed induce organ damage or act as a protectant, through increased ROS formation or mast cell stabilization respectively. Degranulation of mast cells occurs following myocardial ischaemia/reperfusion leads to an interstitial increase in chymase (Zheng *et al.*, 2014) and renin concentration, the latter contributing to activation of the local cardiac renin–angiotensin system. Indeed, mast cell modulators (Jaggi *et al.*, 2007), including adenosine receptor agonists (Rork *et al.*, 2008), reduced ischaemia/reperfusion injury. p66Shc limits the basal release of granule contents from mast cells by inhibiting microvesicle budding from the plasma membrane through regulation of mast cell actin dynamics (Masi *et al.*, 2014).

- Does p66Shc activation contribute to endogenous organ protective interventions such as pre-conditioning, post-conditioning and remote conditioning *in vivo*?

The involvement of p66Shc in endogenous protection induced by ischaemic preconditioning has been suggested from studies on neuronal cells (see discussion earlier). However, *in vivo* studies and studies in other organs – such as the heart – are lacking.

Again, one interesting observation comes from studies in pig hearts *in vivo* in which the reduction in infarct size induced by ischaemic preconditioning was attenuated by pretreatment with ascorbic acid. This loss of protection might involve scavenging of ROS during the preconditioning ischaemia/reperfusion episode (Skyschally *et al.*, 2003). Interestingly, p66Shc-mediated ROS up-regulation was significantly decreased in the presence of ascorbic acid, which decreases p66Shc expression by increasing its ubiquitination (Kirmani *et al.*, 2013).

- Does inhibition of p66Shc activation following prolonged ischaemia/reperfusion present a new therapeutic option to reduce irreversible tissue injury?

As detailed above, data on p66Shc knockout suggest that organ damage following ischaemia/reperfusion might be reduced. However, data on pharmacological interventions modifying p66Shc expression and/or activity are sparce and need to be extended. Furthermore, existing pharmacological interventions for cardiac protection should be revisited to investigate their effect on p66Shc expression and activity.

Conclusion

The evidence so far reported and herein discussed supports the concept that pharmacological modulation of p66Shc expression and activity may be an effective target for the treatment of atherosclerotic vascular disease, as well as a means of modifying the effects of hypertrophic, inflammatory and neurohormonal stimuli in the overloaded heart. The importance of p66Shc for ischaemia/reperfusion injury in the heart, as well as in the brain, requires further elucidation, and the role of p66Shc in the signalling cascade of endogenous protective mechanisms needs to be clarified.

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Conflict of interest

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