The cellular prion protein counteracts cardiac oxidative stress

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1. Introduction

The cellular prion protein (PrPc) is a highly conserved sialoglycoprotein present in most mammalian cells, which is attached to the cell surface via a glycolipid anchor. Upon a conformational change towards a β-enriched structure, PrPc converts into an aberrant isoform (PrPSc). PrPSc is the major constituent of prions, the aetiologic agents of a group of rare and incurable neurodegenerative disorders, named transmissible spongiform encephalopathies (TSEs) or prion diseases, which affect both humans and animals.1,2 Neurodegeneration is the hallmark of prion diseases. However, it has been shown that cardiac muscles can also be naturally affected during TSEs. Examples of this kind are the prion deposits observed in the myocardium of a severe dilated cardiomyopathy area of patients and animal models showed increased PrPc levels.14–16 A role for PrPc in the protective adaptive response against oxidative stress was also suggested by the reduced or exacerbated ischemic

Knowledge on PrPc physiological function would help elucidating the mechanism of prion pathogenesis. However, the absence of gross phenotypic traits in PrP-knockout (PrP-KO) mice has hampered so far full understanding of the role of the protein. A compensatory mechanism could explain the lack of overt PrP-KO phenotypes, whereby changes linked to PrPc deficiency would become apparent only under defined stress conditions.9 Nonetheless, use of several strategies—recombinant PrP forms, cell model systems, and genetically modified animals—has proposed a wide spectrum of PrPc roles, ranging from differentiation to cell survival,10,11 and, in particular, to cell protection from oxidative injury.12 For example, after (transient or permanent) cerebral ischemia involving oxidative stress and cell death,13 the affected brain area of patients and animal models showed increased PrPc levels.14–16

Aims

The cellular prion protein, PrPc, whose aberrant isoforms are related to prion diseases of humans and animals, has a still obscure physiological function. Having observed an increased expression of PrPc in two in vivo paradigms of heart remodelling, we focused on isolated mouse hearts to ascertain the capacity of PrPc to antagonize oxidative damage induced by ischaemic and non-ischaemic protocols.

Methods and results

Hearts isolated from mice expressing PrPc in variable amounts were subjected to different and complementary oxidative perfusion protocols. Accumulation of reactive oxygen species, oxidation of myofibrillar proteins, and cell death were evaluated. We found that overexpressed PrPc reduced oxidative stress and cell death caused by post-ischaemic reperfusion. Conversely, deletion of PrPc increased oxidative stress during both ischaemic preconditioning and perfusion (15 min) with H2O2. Supporting its relation with intracellular systems involved in oxidative stress, PrPc was found to influence the activity of catalase and, for the first time, the expression of p66Shc, a protein implicated in oxidative stress-mediated cell death.

Conclusions

Our data demonstrate that PrPc contributes to the cardiac mechanisms antagonizing oxidative insults.

Keywords

Cellular prion protein • PrP • Oxidative stress • Heart • ROS
damage observed in the brains of animals overexpressing PrP\textsuperscript{C} or KO for PrP\textsuperscript{C}, respectively. In light of these results and the knowledge that skeletal and cardiac muscles from PrP-KO mice display higher levels of oxidized lipids and proteins, and reduced antioxidant activity than the wild-type (WT) counterparts, we investigated whether PrP\textsuperscript{C} protects the heart from oxidative stress in analogy to brains. The study was carried out by comparing hearts isolated from mice expressing different quantities of PrP\textsuperscript{C}, WT and PrP-KO mice, and mice with a three-fold expression of PrP\textsuperscript{C} (PrP-OE). After applying different, and complementary, ischaemic and non-ischaemic oxidative stress protocols, hearts were examined for the accumulation of reactive oxygen species (ROS), oxidation of myofibrillar proteins, and cell death. To unravel the molecular basis of PrP\textsuperscript{C} antioxidant action, we also analysed proteins involved in heart antioxidant responses. The obtained results indicate that PrP\textsuperscript{C} belongs to the cell mechanisms protecting cardiomyocytes from oxidative insults, possibly by influencing the activity of catalase and the expression of p66\textsuperscript{Shc}. Of interest, a possible in vivo relevance of PrP\textsuperscript{C} against heart disease was suggested by the increased PrP\textsuperscript{C} expression elicited in two animal models of heart remodelling (HR) towards heart failure implicating oxidative stress.

2. Methods

See Supplementary material online for a more detailed description.

2.1 Animals

All animal experimentation conformed to the Guide for the Care and Use of Laboratory Animals (US National Institute of Health, publication no. 85–23, revised 1996). We used 12-month-old male Chinchilla rabbits for rapid left ventricular pacing, and 3-month-old male C57BL/6 mice for permanent coronary artery ligation provoking myocardial infarction. For all other experiments, we used WT mice with FVB genotype and congenic PrP-KO (line F10), and PrP-OE mice (line Tg37) (both lines kindly provided by the MRC Prion Unit, London, UK).\textsuperscript{8} The PrP-KO line was obtained by cross-breeding for 10 generations Zurich I PrP-KO mice (bearing a hybrid SV129X/C57-Bl6 genotype)\textsuperscript{8} with FVB WT (PrP\textsuperscript{+}+/+) mice, and then by interbreeding the PrP\textsuperscript{+}+ littermates (N10) to generate the PrP-KO (F10) line with an almost pure (theoretically >99.9\%) FVB genotype.\textsuperscript{8} PrP-OE Tg37 mice were generated by reintroducing PrP transgenes onto the PrP-KO F10 line, in which the three-fold expression of PrP\textsuperscript{C} did not form prion-like aggregates.\textsuperscript{8} To minimize individual biological variability, 4-month-old male mice were always used. Before thoracotomy, rabbits subjected to left ventricular pacing were anaesthetized by means of i.v. administration of ketamine (50 mg/kg)–xylazine (3 mg/kg), followed by propofol (12–25 mL/h)–fentanyl (0.003 mg/kg). Before instrumentation for myocardial infarction, mice were anaesthetized with inhalation of 1.5% isofluorane mixed with pure oxygen, intubated, and ventilated with the same gas mixture during the entire procedure. For ex vivo heart perfusion experiments, following anaesthesia by intraperitoneal injection of a cocktail of tiletamine hydrochloride and zolezepam hydrochloride (Zoletil 100, 30 mg/kg body weight; Virbac, Milan, Italy), mice were euthanized by cervical dislocation before heart excision.

2.2 Induction of myocardial infarction in mice

The myocardial infarction model in mice was based on the in vivo permanent occlusion of the left anterior descending coronary artery, as described.\textsuperscript{22} The permanent occlusion was kept for 24 h, 1, 2, 4, and 6 weeks, and for each time point, sham-operated mice were used as a reference. At the end of observation, animals were sacrificed, and hearts were explanted and stored at −80°C for subsequent analyses.

2.3 Perfusion protocols of isolated mouse hearts

Perfusion of isolated hearts was carried out in the non-recirculating Langendorff mode,\textsuperscript{23} with a perfusion buffer (PB) containing (in mM) 115.0 NaCl, 4.75 KCl, 2.15 KH\textsubscript{2}PO\textsubscript{4} (pH 7.4), 25.0 NaHCO\textsubscript{3}, 0.65 MgSO\textsubscript{4}, 1.69 CaCl\textsubscript{2}, and 11.0 glucose, and gassed with O\textsubscript{2} (95\%) and CO\textsubscript{2} (5\%). After a 5-min perfusion stabilization period, hearts were subjected to the following perfusion protocols: (i) ischaemia/reperfusion (I/R), consisting of 40 min of global ischaemia (achieved by stopping the coronary flow) followed by 15 min of reperfusion; (ii) ischaemia preconditioning (IPC), i.e. three cycles of 5-min ischaemia and 5-min reperfusion, followed, or not, by I/R; and (iii) perfusion (for 15, or 30 min) with hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}, 1 mM in PB). Control (normoxic) hearts were subjected only to the 5-min stabilization protocol. For subsequent analyses, 5 mL of samples of the coronary effluent were collected at 1-min intervals during post-ischaemic reperfusion, or perfusion with H\textsubscript{2}O\textsubscript{2}, as described.\textsuperscript{24} At the end of the experiments, hearts were either immediately used for determining the total heart content of lactic dehydrogenase (LDH), and dihydroethidium (DHE) staining, or stored in liquid nitrogen for further tests.

2.4 Estimation of enzymatic activity

2.4.1 Lactic dehydrogenase

Myocardial cell death following the different treatments was quantified by the presence, i.e. the activity, of LDH in the coronary effluent, as described.\textsuperscript{24}

2.4.2 Superoxide dismutase

Superoxide dismutase 1 (SOD1) (Cu/Zn–SOD) and SOD2 (Mn–SOD) activities were determined on heart cytosolic and mitochondrial fractions, respectively, by estimating the inhibition of (SOD-containing) heart samples on xanthine/xanthine oxidase-induced reduction of cytochrome c.\textsuperscript{25} Data were normalized to those obtained with 1 U of purified SOD, and reported as the percentage of the mean value obtained with WT samples.

2.4.3 Catalase

Catalase (CAT) activity of homogenized heart samples was measured by following H\textsubscript{2}O\textsubscript{2} consumption at 240 nm.\textsuperscript{26} Data were calibrated by means of a standard curve generated by using known amounts of purified CAT, and expressed as the percentage of the mean value obtained with WT samples.

2.5 Western blotting and densitometric analysis

After determining total protein contents, Western blot (WB) analyses on whole heart homogenates, or mitochondrial fractions, were carried out by SDS–PAGE protein separation under either non-reducing (for mouse PrP\textsuperscript{C} and tropomyosin (TM)), or reducing (for rabbit PrP\textsuperscript{C}, p66\textsuperscript{Shc}, SOD1, and SOD2) protocols: (i) ischaemia/reperfusion (I/R), consisting of 40 min of global ischaemia (achieved by stopping the coronary flow) followed by 15 min of reperfusion; (ii) ischaemia preconditioning (IPC), i.e. three cycles of 5-min ischaemia and 5-min reperfusion, followed, or not, by I/R; and (iii) perfusion (for 15, or 30 min) with hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}, 1 mM in PB). Control (normoxic) hearts were subjected only to the 5-min stabilization protocol. For subsequent analyses, 5 mL of samples of the coronary effluent were collected at 1-min intervals during post-ischaemic reperfusion, or perfusion with H\textsubscript{2}O\textsubscript{2}, as described. Data were calibrated by means of a standard curve generated by using known amounts of purified CAT, and expressed as the percentage of the mean value obtained with WT samples.

2.5.1 Antibodies

The following mono- (m) and polyclonal (p) antibodies (Ab) were used (dilutions are indicated in parenthesis): anti-PrP mouse mAb BH4 (1 : 7000, a kind gift of Dr M.S. Sy, Case Western University, Cleveland, OH, USA); anti-TM
mouse mAb CH1 (1:2000) (Sigma); anti-Shc rabbit pAb (1:2000) (Transduction Laboratories); anti-SOD1 rabbit pAb (1:1000) (Abcam); anti-SOD2 rabbit pAb (1:2000) (Sigma); and anti-SDHA rabbit pAb (1:1000) (Sigma).

2.6 In situ superoxide detection

Tissue staining with DHE was used to measure heart accumulation of the superoxide anion.\(^{27}\) Freshly prepared heart cryosections (10 \(\mu\)m) were incubated (30 min, 37 °C) in the presence of DHE (10 \(\mu\)M in DMSO), and digitalized fluorescence images were subjected to an automatic computer-based analysis. The fluorescence intensity of each tissue section was calculated as the average intensity from four randomly selected fields, and then normalized to the mean value of the WT samples.

2.7 Statistical analysis

All results are presented as mean ± standard error of the mean (SEM). The number of replicates, \(n\), for each set of results is reported in the figure legends, and refers to independent experiments, i.e. measurements performed on different hearts. Statistical analysis for pair comparison within groups was performed using the Student’s \(t\)-test, whereas analysis between groups by one-way ANOVA followed by the Bonferroni’s post hoc test for group differences. Statistics were computed with the Prism software (GraphPad Software, San Diego, CA, USA). Differences between means were accepted as statistically significant at the 95% level (\(P < 0.05\)).

3. Results

3.1 PrP\(^C\) expression is transiently increased in models of HR towards failure

Initially, we verified whether hearts responded to ischaemic injury by increasing the expression of PrP\(^C\), as was previously observed in ischaemic brains.\(^{14–16}\) To this end, we analysed by WB already available samples from mice with permanent coronary artery ligation. The finding that PrP\(^C\) was transiently increased in the initial phase of HR (Figure 1 and see Supplementary material online, Figure S1) suggests that the protein acts as an early adaptive cellular response. This concept was supported by the augmented PrP\(^C\) content observed in a non-ischaemic HR model (rabbits subjected to rapid left ventricular pacing, see Supplementary material online, Figure S2).

Notably, although both the above in vivo models involve oxidative damage,\(^{28–30}\) they are not suited to establish a direct relationship between a given protein, e.g. PrP\(^C\), and cardiac oxidative stress. We thus investigated this issue by comparing I/R damage in hearts isolated from WT mice with genetically modified congenic mice, i.e. PrP-KO mice and a transgenic PrP-OE line. We first controlled by WB the expression of PrP\(^C\) in the different hearts (Figure 2; see also Supplementary material online, Figure S3). Though to a lesser extent than in the brain (Figure 2, lane 1), PrP\(^C\) was readily identified in WT hearts (lane 2), while higher amounts were present in PrP-OE hearts (lane 4) (OE to WT PrP\(^C\) ratio, 3.0 ± 0.2; see also Supplementary material online, Figure S4). Of interest, the increase of PrP\(^C\) expression genetically determined in PrP-OE hearts is similar to the increase observed in WT hearts (Figure 2, lane 3), while higher amounts were present in PrP-OE hearts (lane 4) (OE to WT PrP\(^C\) ratio, 3.0 ± 0.2; see also Supplementary material online, Figure S4). Of interest, the increase of PrP\(^C\) expression genetically determined in PrP-OE hearts is similar to the increase observed in WT hearts (Figure 2, lane 3). The three PrP\(^C\) bands evident in Figure 2 reflect the typical glycosylation of the protein, whose different glycoforms (non-, mono-, or di-glycosylated with increasing molecular mass) correspond to the variable occupancy of the two N-glycosylation sites.\(^{1}\) A link between the biological function and glycosylation states of PrP\(^C\) is still missing, yet the presence of the fully glycosylated form indicates the correct post-translational processing of the protein in both WT and PrP-OE hearts (lanes 2 and 4).

3.2 Isolated PrP-OE hearts are protected against ROS accumulation, protein oxidation, and cell death induced by post-ischaemic reperfusion

In the I/R protocol, hearts were subjected to a 40-min period of ischaemia followed by 15 min of reperfusion, after which death of cardiomyocytes was monitored by quantifying the release of LDH in the coronary effluent. As shown in Table 1 (first line), and in the bar diagram of Figure 3A, I/R produced ~27% loss of cell viability in both WT and PrP-KO hearts, in contrast to the significantly reduced cell death (~22%) in PrP-OE hearts, indicating that the three-fold-overexpressed PrP\(^C\) protected from I/R injury (\(F_{2,108} = 3.249, P < 0.05\); post hoc test: \(P < 0.05\) for PrP-OE vs. WT and vs. PrP-KO). The protection afforded by PrP\(^C\) overexpression was further supported by evaluating the area of necrosis by triphenyl tetrazolium chloride staining of heart slices in a limited number of experiments (see Supplementary material online, Figure S5).
Although prolonged no-flow ischaemia of isolated hearts provokes a complex set of cellular derangements (e.g. mitochondria dysfunctions, ATP depletion, pH changes, and impairment of ion homeostasis), a major contribution to heart injury comes from the oxidative damage caused by massive ROS amounts produced upon re-establishing the coronary flow. To provide evidence for the antioxidant capacity of PrP\textsuperscript{C}, we measured both ROS generated by hearts at the end of the I/R protocol, and the ROS-induced oxidation of heart contractile proteins. Of these, we analysed TM, in light of the link between derangement of heart contractility and quantity of TM dimers that form after the cross-linking of single cysteine residues present in TM molecules.

By staining heart cryosections with DHE, whose reaction with superoxide anions generates the fluorogenic 2-hydroxyethidium, and by immunoblot experiments of heart samples probed with an antibody onary flow. To provide evidence for the antioxidant capacity of PrPC, we thus investigated these two opposite faces of ROS. First, we measured both ROS generated by hearts at the end of the I/R protocol (Figure 3B; \( F_{2,12} = 10.470, P < 0.005; \) post hoc test: \( P < 0.01 \) PrP-OE vs. WT, \( P < 0.001 \) PrP-OE vs. PrP-KO), and generated significantly less TM dimers (Figure 3C; \( F_{2,11} = 4.291, P < 0.05; \) post hoc test: \( P < 0.05 \) for PrP-OE vs. WT and vs. PrP-KO; see also Supplementary material online, Figure S6), compared with the other two groups. An almost undetectable fluorescence signal was instead displayed by normoxic heart cryosections (data not shown).

Taken together, these data indicate that only the overexpression of PrP\textsuperscript{C} protects the heart from I/R oxidative damage. To explain why normal amounts of PrP\textsuperscript{C} could not antagonize I/R injury, and why mortality was similar in WT and PrP-KO hearts, one should consider the aggressiveness of the entire I/R procedure on cardiomyocytes, given that post-reperfusion ROS act synergistically with the complex set of the above-mentioned cell derangements provoked by prolonged ischaemia.

### 3.3 PrP\textsuperscript{C} antagonizes the effects of ROS in isolated hearts

ROS are known to be involved both in cell damage and in cell signalling and protection. To better understand the antioxidant property of PrP\textsuperscript{C}, we thus investigated these two opposite faces of ROS. First, we used IPC, involving protective ROS signalling; secondly, we applied a severe and direct oxidative stress by means of perfusion with H\textsubscript{2}O\textsubscript{2}.

IPC, which consists of repetitive brief episodes of I/R preceding a prolonged ischaemic period, is suggested to produce sub-lethal ROS that trigger defence mechanisms eventually antagonizing the large burst of ROS during post-ischaemic reperfusion. DHE staining of hearts at the end of three cycles of short I/R episodes (5 min/5 min) showed that PrP-KO hearts accumulated significantly higher ROS compared with WT and PrP-OE hearts (Figure 4A; \( F_{2,11} = 7.755, P < 0.01; \) post hoc test: \( P < 0.01 \) PrP-KO vs. WT, \( P < 0.05 \) PrP-KO vs. PrP-OE). These data indicate that PrP\textsuperscript{C} antagonize IPC-induced ROS accumulation. However, the larger ROS amount generated in the absence of PrP\textsuperscript{C} was unable to increase the protection observed in WT hearts (Table 1, compare the first and second line, and Figure 4B, compare grey and hatched bars, \( P < 0.01 \) for both WT and PrP-KO hearts, Student’s \( t \)-test). Conversely, no significant effect was observed in PrP-OE hearts, which displayed ROS levels comparable with those of the WT group. Addition of the potent ROS scavenger, N-2-mercaptopropionyl-glycine (MGP), abolished the effect irrespective of the PrP genotype (third line of Table 1; black bars of Figure 4B), confirming that (mild) production of ROS during IPC is necessary for heart protection.

Next, a more direct evidence of PrP\textsuperscript{C}’s antioxidant potential was obtained using a model (perfusion with H\textsubscript{2}O\textsubscript{2}) in which myocardial damage can be ascribed primarily to oxidative stress. Figure 5A shows that damage by H\textsubscript{2}O\textsubscript{2} was initially small and that it became more evident with longer exposure to H\textsubscript{2}O\textsubscript{2} in all hearts. However, a clear PrP-KO phenotype emerged at later time points of perfusion (14 and 15 min), when there was both a significantly higher cell death [fourth

<table>
<thead>
<tr>
<th>LDH release (%)</th>
<th>WT</th>
<th>PrP-KO</th>
<th>PrP-OE</th>
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<tbody>
<tr>
<td>I/R</td>
<td>27.2 ± 2.1 (( n = 38 ))</td>
<td>27.2 ± 1.8 (( n = 38 ))</td>
<td>21.7 ± 1.4 (( n = 35 ))</td>
</tr>
<tr>
<td>IPC + I/R</td>
<td>18.3 ± 2.4 (( n = 14 ))</td>
<td>15.4 ± 2.4 (( n = 12 ))</td>
<td>17.5 ± 2.0 (( n = 9 ))</td>
</tr>
<tr>
<td>IPC (+ MGP) + I/R</td>
<td>26.4 ± 4.9 (( n = 4 ))</td>
<td>22.5 ± 4.2 (( n = 4 ))</td>
<td></td>
</tr>
<tr>
<td>H\textsubscript{2}O\textsubscript{2}</td>
<td>6.2 ± 1.1 (( n = 11 ))</td>
<td>12.5 ± 2.3 (( n = 11 ))</td>
<td>6.5 ± 2.2 (( n = 11 ))</td>
</tr>
</tbody>
</table>

Loss of myocardial viability of WT, PrP-KO, and PrP-OE hearts (second, third, and fourth columns, respectively) was evaluated as the percentage of LDH released in the coronary effluent over heart total (coronary effluent plus tissue homogenate) LDH, following perfusion with the protocol indicated in the first column. Data are mean ± SEM; \( n \) is the number of experiments. For statistical analysis, see the text and the legends of Figures 3–5. Other details are described in Methods and also in Supplementary material online.

IPA, ischaemia followed by reperfusion; IPC, ischaemic preconditioning before I/R; MGP, N-2-mercaptopropionyl-glycine.
Table 1; and Figure 5A; (100 ± 35)% relative increase with respect to WT hearts after 15 min perfusion; for statistical analysis, see Supplementary material online, Table S1), and TM oxidation (Figure 5B; $F_{2,22} = 4.75$, $P < 0.05$; post hoc test: $P < 0.05$ for PrP-KO vs. WT, $P < 0.01$ for PrP-KO vs. PrP-OE), than in PrP$^+$-expressing samples.

As shown (Figure 5A, black line), MPG fully reversed the PrP-KO phenotype.

Extending the perfusion to 30 min resulted in exacerbated injury of all hearts. However, while there was no difference in death between WT and PrP-KO cardiomyocytes, PrP$^+$ overexpression significantly
protected the myocardium (inset in Figure 5A). These results further support our suggestion that cells that had been impaired by a protracted ischaemic episode (Figure 3A), or, as in this case, by prolonged perfusion with H₂O₂, need higher PrP C amounts to prevent ROS-mediated cell death.

3.4 Molecular mechanisms of PrP C antioxidant function in hearts: catalase activity and p66Shc expression

Because of previous indications that PrP C modulates various cell antioxidant systems, 12,21 we investigated whether the response to...
detoxification from H₂O₂ is well recognized, and reduced CAT activity to variations of myocardial anti-/pro-oxidant resources. 

oxidative insults in hearts with different PrP⁰ levels could be attributed to variations of myocardial anti-/pro-oxidant resources.

We first tested the activity of CAT. The importance of CAT in the cell detoxification from H₂O₂ is well recognized, and reduced CAT activity has already been reported in PrP-KO hearts compared with WT hearts.²¹ In our case, we observed a significant decrease of CAT activity (~15%) in PrP-KO hearts compared with PrP²⁰-expressing counterparts, but no significant difference between WT and PrP-OE hearts (Figure 6A; F₂,27 = 4.742, P < 0.05; post hoc test: P < 0.05 for both PrP-KO vs. WT and PrP-KO vs. PrP-OE). However, we could not relate this finding to a reduced expression of the enzyme, as CAT was not detected by WB, possibly because of its low expression in the cardiac tissue.²⁴

We also tested SOD, another fundamental component of ROS-scavenging systems in cells. SOD exists in three isoforms, of which one resides mainly in the cytosol (Cu/Zn-dependent SOD1) and another in mitochondria (Mn-dependent SOD2). A reduction in total SOD activity was reported in PrP-KO and in prion-infected brains,²¹,²⁵ but these data were questioned.²⁶,²⁷ Using cytosolic and mitochondrial fractions to evaluate separately the activity of SOD1 and SOD2, we found that both enzymes displayed similar activity (Figure 6A) and expression level (data not shown) in all hearts.

To investigate a further link between PrP²⁰ and intracellular mechanisms involving oxidative stress, we considered p66Shc, a splice variant of the two cytosolic adaptor proteins p52Shc and p46Shc. This choice stemmed from the notion that mitochondria are one of the major cell sources of ROS, and that p66Shc is a key factor in ROS formation because, after re-localizing to the mitochondrial intermembrane space, it diverts electrons from the respiratory chain to O₂⁻.²⁸ Accordingly, embryonic fibroblasts and isolated hearts from p66Shc-KO mice have been shown to be more resistant to apoptotic stimuli and I/R injury, respectively.²⁴,²⁹ As shown in the WB analysis of Figure 6B, PrP-KO total heart homogenates contained higher levels (~80%) of p66Shc than PrP²⁰-expressing samples (F₂,43 = 3.293, P < 0.05; post hoc test: P < 0.05 for both PrP-KO vs. WT and PrP-KO vs. PrP-OE). Conversely, all hearts displayed similar amounts of p52Shc and p46Shc (data not shown). Most importantly, in spite of the finding that PrP²⁰ ablation did not affect the heart mitochondrial content (assessed by the WB quantification of the mitochondrial marker SDH, data not shown), we found that the mitochondrial fraction of PrP-KO hearts had also higher (~60%) p66Shc content than PrP²⁰-expressing hearts (Figure 6C; F₂,13 = 6.989, P < 0.01; post hoc test: P < 0.05 for PrP-KO vs. WT, and P < 0.01 for PrP-KO vs. PrP-OE).

4. Discussion

By using for the first time in the prion field perfused ex vivo hearts to study PrP²⁰ antioxidant features, the present work provides evidence that PrP²⁰ modulates cardiac ROS formation, and that its overexpression protects the heart from reperfusion injury by reducing oxidative stress.

A close insight into PrP²⁰ antioxidant features was accomplished by comparing the response of hearts isolated from mice with different PrP genotypes (WT, OE, and KO) to three complementary perfusion protocols—I/R, preceded or not by IPC, and perfusion with H₂O₂. Taken together, these models highlighted a likely physiological significance of PrP²⁰ antioxidant properties under stress conditions.

We found that, while ischemia (40 min) followed by reperfusion (15 min) failed to underscore differences in cell death between WT and PrP-KO hearts, PrP-OE hearts showed a significantly reduced cell damage and lower amounts of ROS and oxidized myofibrillar proteins. This observation is in line with the finding that high quantities of (endogenous or exogenous) PrP²⁰ defend against ischemic brain injury and hypoxia.¹⁵,¹⁷ It is therefore not surprising the inability of...
physiological PrPC levels to oppose the fierce post-ischaemic burst of ROS, which could account for the lack of major differences in I/R injury between WT and PrP-KO hearts.

A further insight into the protective property of PrPC came from perfusion with H2O2, an oxidative challenge devoid of ischaemic insults, which should set at best the conditions for appreciating the specific contribution of PrPC to the antioxidant defences of the heart. Indeed, the overt PrP-KO phenotype disclosed by 14–15 min H2O2-based perfusion and the similar response of WT and PrP-OE hearts—with a lower extent of cell death and oxidized proteins—supports the notion that physiological PrPC amounts are sufficient to protect from oxidative insults. PrPC anti-ROS properties in an intact tissue are further corroborated by the response to longer (30 min) exposure to H2O2, after which the oxidative damage to cardiomyocytes must have been so profound that only overexpressed PrPC could partly alleviate ROS injury.

Another indication of the antioxidant role of PrPC was obtained by applying IPC, which induced a larger production of ROS in hearts from PrP-KO mice. However, this larger ROS formation did not improve IPC protection, in line with the notion that no intervention is able to increase the cardioprotective efficacy of IPC. Nor could IPC add further protection to that already exerted by three-fold levels of PrPC in PrP-OE hearts, suggesting that IPC and PrPC overexpression work via the same mechanism, i.e. by reducing ROS formation upon reperfusion.

Taken together, these results indicate the capacity of PrPC to antagonize ROS under a variety of circumstances: when ROS provoke heart injury (as after I/R- and H2O2-based protocols), or trigger intracellular signalling (as during IPC). Besides the acute model of perfused hearts highlighting a direct action of PrPC on ROS, one can also envisage adaptive mechanisms in which an increased expression of PrPC—as observed here in two (ischaemic and non-ischaemic) in vivo models of HR and previously in cerebral ischaemia—strengthens the endogenous defence of the heart against oxidative stress.

To identify the molecular basis of PrPC protection, we first considered CAT and SOD, whose activity was previously reported to be reduced in different PrP-KO cells and tissues, including heart. We confirmed the significant decrease of CAT activity in PrP-KO hearts compared with PrPC-expressing hearts. However, because we failed to detect the protein by WB, further studies are needed to clarify whether such a reduction is consequent to a decreased quantity, or an inhibitory modification, of the enzyme.

Figure 6 Catalase activity is reduced, and p66Shc expression is increased, in PrP-KO hearts. (A) Samples of untreated hearts from WT (grey bars), PrP-KO (hatched bars), and PrP-OE (black bars) mice were tested for CAT, SOD1, and SOD2 activities. Whereas in all hearts a similar SOD1 and SOD2 activity is found, the activity of CAT is significantly reduced (~15%) in PrP-KO hearts with respect to PrPC-expressing hearts. (B and C) p66Shc content of total homogenates (B), or mitochondrial fractions (C), of untreated hearts was assessed by WB. Upper panels show representative WBs (out of 5 to 18 independent experiments) for p66Shc. Lower panels report the bar diagrams of the densitometric analysis of p66Shc-immunoreactive bands (expressed as the percentage of WT samples), indicating that p66Shc is significantly more abundant in both total homogenates (~80%) and mitochondrial fractions (~60%) of PrP-KO hearts than in WT and PrP-OE samples. In (A), n = 12 for WT, n = 8 for PrP-KO, and n = 10 for PrP-OE, for CAT activity; n = 4 for each PrP genotype, for SOD1 and SOD2 activities; in (B): n = 15 for WT, n = 18 for PrP-KO, and n = 13 for PrP-OE; in (C): n = 6 for WT, and n = 5 for PrP-KO and PrP-OE. *P < 0.05, **P < 0.01, Bonferroni’s post hoc test. Further details are described in Methods and Supplementary material online.
In contrast, hearts with different PrP genotypes displayed similar activities and amounts of both cytosolic and mitochondrial SOD isofoms. SOD regulation by PrP<sup>C</sup> has been highly debated, and some authors have rejected this possibility on methodological grounds. To note that, we tested SOD activity with the xanthine/xanthine oxidase-based assay, because the sensitivity and reproducibility of the test are higher than the alternative nitro-blue tetrizolium-based technique.

However, we also provided evidence that PrP<sup>C</sup>-dependent defence against ROS may involve control of p66<sup>Shc</sup> expression. Following its partial redistribution from the cytosol to the intermembrane space of mitochondria, p66<sup>Shc</sup> oxidizes cytochrome c and the resulting electrons reduce O<sub>2</sub> to H<sub>2</sub>O<sub>2</sub>. This is a powerful mechanism that produces as much as one-third of the H<sub>2</sub>O<sub>2</sub> pool of cells. Accordingly, p66<sup>Shc</sup>-KO cells are protected against oxidative stress, and, in particular, p66<sup>Shc</sup>-KO hearts are more resistant to I/R injuries. Hence, our finding that PrP-KO hearts expressed higher amounts of both total and mitochondrial p66<sup>Shc</sup> could not only explain the lower capacity of PrP-KO hearts to antagonize ROS effects, but also add a new actor (PrP<sup>C</sup>) in p66<sup>Shc</sup>-dependent oxidative stress and mitochondria-mediated apoptotic mechanisms.

By proposing that PrP<sup>C</sup> is a part of signal transduction complexes in which the protein interacts with several partners, a few studies have provided the mechanistic basis to explain how PrP<sup>C</sup> could modulate intracellular events in spite of its location at the cell surface. By controlling different signalling pathways, including those mediated by Ca<sup>2+</sup>, Erk 1/2, or PI3K/Akt, PrP<sup>C</sup> could ultimately also affect gene expression, as suggested by different approaches, including large-scale proteomics. Interestingly, the recent finding that activation of Akt in the heart induces the nuclear E2-related factor 2 (Nrf2), and transcription of cytoprotective genes against I/R injury, provides a possible connection between PrP<sup>C</sup> and the control of antioxidant systems. Indeed, this mechanism could help explaining why PrP-KO hearts display a reduced activity of CAT, whose expression is under Nrf2 control. However, additional mechanisms have to be identified to explain the change in p66<sup>Shc</sup>, which—to our knowledge—has never been reported to be controlled by the Nrf2 pathway.

In addition, PrP<sup>C</sup>-dependent up-regulation of anti-(CAT), and down-regulation of pro-(p66<sup>Shc</sup>), oxidant systems cannot fully account for the observed antagonism of PrP<sup>C</sup> against ROS injury, given that overexpressed PrP<sup>C</sup> protects hearts from I/R and 30-min H<sub>2</sub>O<sub>2</sub>-induced injuries, but leaves unaltered both the activity of CAT and the expression of p66<sup>Shc</sup> with respect to WT hearts. This finding thus entails the existence of other sufficiently strong (PrP<sup>C</sup>-dependent) protective mechanisms that counteract the severe damage provoked by I/R and prolonged H<sub>2</sub>O<sub>2</sub> treatment.

5. Conclusions

In conclusion, we have provided evidence that, following prolonged I/R, overexpression of PrP<sup>C</sup> reduces oxidative stress through an as yet unknown mechanism, and protects the heart from irreversible injury. On the other hand, abrogation of PrP<sup>C</sup> expression potentiated ROS formation during the preconditioning phase of IPC, and aggravated the oxidative damage and cell death caused by perfusion with H<sub>2</sub>O<sub>2</sub>. The protective effects of PrP<sup>C</sup> are likely to be implemented also in vivo, as shown by the increased expression of the protein in two models of HR, suggesting that cardiac PrP<sup>C</sup> might contribute to endogenous defences against oxidative stress. Further studies, however, are needed to fully elucidate the entire array of mechanisms underlying the antagonistic capacity of PrP<sup>C</sup> in the heart, and, possibly, in other organs.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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