Research article

Putative proto-oncogene *Pir* expression is significantly up-regulated in the spleen and kidney of cytosolic superoxide dismutase-deficient mice

Kamil Brzóska[1], Tomasz M Stępkowski[1], Marcin Kruszewski[1,2]

[1]Institute of Nuclear Chemistry and Technology, Centre for Radiobiology and Biological Dosimetry, Warsaw, Poland, [2]Institute of Agricultural Medicine, Independent Laboratory of Molecular Biology, Lublin, Poland

Iron binding protein pirin was isolated as an interactor of the NFIX transcription factor but it can also form complexes with Bcl3 and NF-κB1(p50). Alterations of pirin expression were observed in various tumors and after exposure to pro-carcinogenic oxidative stressors. The aim of the present work was to study the level of pirin transcription in an *in vivo* model of oxidative stress, namely, in Sod1-deficient mice. We have found that *Sod1*$^{-/-}$ mice have a significantly elevated level of *Pir* mRNA in the spleen and kidney but not in the liver, heart, or/and brain. We have also shown that similarly to its human ortholog, the mouse *Pir* gene transcription level varies significantly between organs. The highest expression was found in the liver and the lowest in the spleen and kidney. Based on literature data, we propose the involvement of Nrf2, AP-1, and NF-κB transcription factors in *Pir* up-regulation in *Sod1*$^{-/-}$ mice.

Keywords: NF-κB, Nrf2, Oxidative stress, Pirin, Superoxide dismutase

Introduction

Pirin is a nuclear protein that was isolated and characterized in the human as an interactor of the NFIX transcription factor.[1] Subsequently, it was revealed that pirin also forms complexes with Bcl3 and NF-κB1 (p50) and therefore, may be implicated in the regulation of the NF-κB-related transcription.[2] The crystallographic study of pirin structure showed that the protein binds the iron ion and can be assigned to the functionally diverse cupin superfamily of proteins.[3] In addition to its role as a transcription cofactor, pirin also has enzymatic activity and can oxidize the flavonoid quercetin.[4] Orthologs of pirin have been found in many organisms ranging from Archaea to mammals. The human *PIR* gene is located on chromosome X (Xp22.2) and is expressed at different levels in various organs (heart, brain, liver, kidney, lung, pancreas, placenta, skeletal muscle). The highest expression rates were observed in the liver and heart and the lowest in the brain and pancreas.[1] Some studies have linked pirin orthologs with apoptosis[5,6] and stress responses,[7] whereas other have pointed out the deregulation of pirin expression in several human tumors including acute myeloid leukemia,[8] melanoma,[9,10] and colorectal carcinoma.[11] Several studies reported the up-regulation of *PIR* expression by cigarette smoke in the airway epithelial cells.[5,12,13] Hübner *et al.*[12] identified *PIR* as one of the highly smoking-responsive Nrf2-modulated genes. Nrf2 is an oxidative stress-responsive transcription factor that induces the expression of a battery of detoxifying and antioxidant genes. It binds to specific DNA sequences in the gene's regulatory regions called the antioxidant response elements (AREs).[14] Four such AREs have been identified in the *PIR* promoter but not all of them are thought to be functionally relevant.[12] The presence of ARE elements in its promoter and its dependence on the Nrf2 transcription factor strongly suggest that pirin function is related to the cellular response to stress (in particular, oxidative stress). This assumption is supported by papers that reported up-regulation of pirin orthologs in response to various stress conditions in Cyanobacteria[7] and to camptothecin-induced lethal damage in the tomato.[6]

The aim of the present work was to study the level of pirin transcription in an *in vivo* model of oxidative stress. As mentioned above, there are various studies

Correspondence to: Kamil Brzóska, Institute of Nuclear Chemistry and Technology, Centre for Radiobiology and Biological Dosimetry, Dorodna 16, 03-195 Warsaw, Poland. Email: k.brzoska@ichtj.waw.pl

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presenting pirin up-regulation in certain tumors and tumorigenic conditions in specific tissues. Our intention was to perform a more general, whole organism study on the transcription of *PIR* in different tissues exposed to chronic, pro-carcinogenic oxidative stress conditions. We believe that the transgenic cytosolic superoxide dismutase (Sod1)-deficient mice constitute a suitable model to study transcriptome alterations in such an environment.

Superoxide dismutase (SOD) catalyzes dismutation of the superoxide radical anion leading to hydrogen peroxide and molecular oxygen formation and constitutes a part of the first line of cellular defense against oxidative stress. There are three SOD isoforms in mammals: cytosolic (SOD1, CuZnSOD), mitochondrial (SOD2, MnSOD) and extracellular (SOD3, EC-SOD).[15] *Sod1*$^{-/-}$ mice are characterized by decreased lifespan, elevated incidence of liver cancer,[16] and increased sensitivity to pro-oxidative agents (e.g. paraquat and ethanol).[17,18] Elevated level of oxidative DNA damage and increased mutation frequency in the liver and kidney of *Sod1*$^{-/-}$ mice are clear hallmarks of chronic oxidative stress present in these organs.[16,19,20] Such stress conditions may trigger activation of several transcription factors, among them Nrf2, AP-1, and NF-κB. Indeed, the increase in NF-κB1 (p50) activity in the kidney of Sod1-deficient mice has recently been reported.[20] Despite the lack of information about Nrf2 and AP-1 activity in *Sod1*$^{-/-}$ mice one can suppose that it is also affected. Because of the postulated pirin involvement in response to stress, its participation in the NF-κB signaling pathway and the presence of ARE elements in its promoter sequence, alterations of pirin expression in *Sod1*$^{-/-}$ mice are highly probable. In the present work, we compared pirin expression in Sod1-deficient mice and in wild-type mice.

The presence of ARE elements in pirin promoter had been reported only in humans. Since this work was focused on studying the expression of the mouse ortholog of *PIR*, we show that potential AREs are also present in the sequence of the mouse *Pir* gene promoter.

Materials and methods

Analysis of the mouse Pir promoter sequence

The sequence upstream of the first exon of the mouse *Pir* gene (gi149361525:160701363–160707613) was analyzed for the presence of ARE elements using Bioedit software.[21]

Animals

A breeder pair of mice (strain B6; 129S7-Sod1^{tm1Leb}) heterozygous for a *Sod1*^{tm1Leb} targeted mutation[22] and their progeny were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Males and females heterozygous for the non-functional *Sod1* allele (*Sod1*+/−) were intercrossed and their progeny were kept at 24–25°C, in 80% humidity with a light–dark cycle of 12 hours. The mice received a standard laboratory diet (Labofeed, Kcynia, Poland) and water *ad libitum*. Genotyping using DNA isolated from mouse tails was performed by PCR analysis according to the protocol provided by The Jackson Laboratory. Mice homozygous for the non-functional *Sod1* allele (*Sod1*$^{-/-}$) and control mice homozygous for the wild-type *Sod1* allele (*Sod1*+/+) at the age of 12 months were used in the study. All the experimental procedures involving animals were approved by the local Ethical Commission.

RNA isolation, reverse transcription, and real-time PCR

Liver, kidney, heart, brain, and spleen were excised immediately after death from mice killed by cervical dislocation, flash frozen in liquid nitrogen, and stored at −75°C. Total RNA was extracted from the tissues using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. One microgram of total RNA was converted to cDNA in a 20 µl reaction volume using High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) following the manufacturer's instructions. After reaction, cDNA was diluted to 100 µl with de-ionized, nuclease-free H_2O. Real-time PCR was performed in 20 µl reaction mixture containing 5 µl of diluted cDNA, 4 µl of de-ionized, nuclease-free H_2O, 10 µl of TaqMan Gene Expression Master Mix (Life Technologies), and 1 µl of TaqMan Gene Expression Assay (Life Technologies) for *Pir* (Assay ID: Mm01721878_m1) or *Actb* (Assay ID: Mm00607939_s1). All reactions were run in duplicate. PCR amplification was carried out using 7500 Real-Time PCR System (Life Technologies) with an initial 10-minute step at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative expression levels were calculated using REST 2009[23] software with *Actb* as a reference control. Significant differences ($P < 0.05$) in relative expression were determined by the pair-wise fixed reallocation randomization test built in REST 2009.[23] In this test Ct values for reference gene (*Actb*) and target gene (*Pir*) were jointly, repeatedly (2000 iterations), and randomly reallocated to control and sample group and the expression ratios were calculated each time. The proportion of expression ratios that were as high as that observed in the experiment gave the *P* value of the test.

Results

Four putative ARE elements are present in the mouse Pir promoter

The analysis of the sequence 6 kb upstream of the first exon of the mouse *Pir* gene allowed us to identify four

Table 1 AREs in the promoter sequence of mice *Pir* gene

ARE core sequence	Position relative to TSS
5′-GCTAAATCAT-3′ 3′-CGATTTAGTA-5′	−5913
5′-GCCAGGTCAT-3′ 3′-CGGTCCAGTA-5′	−2432
5′-GCCAGGTCAT-3′ 3′-CGGTCCAGTA-5′	−214
5′-GCTGAGTCAC-3′ 3′-CGACTCAGTG-5′	+123

The position of each element is marked relative to the transcription start site (according to mouse *Pir* cDNA sequence accession number NM_027153).

putative ARE elements matching ARE consensus 5′-RTGAYNNNGC-3′. The sequences and positions of the identified elements are presented in Table 1.

Pir expression significantly differs between the organs of wild-type mice

The *Pir* mRNA levels in spleen, kidney, brain, heart, and liver of 16 (8 males and 8 females) $Sod1^{+/+}$ mice were estimated by real-time PCR with *Actb* as reference control. The lowest mean expression was found in the spleen and was set as 1. The expression levels in the other organs were compared to the spleen by the ΔΔCt method and were shown as an expression ratio (fold change) over the spleen (Fig. 1). The highest mean expression was found in the liver (64.1 times higher than in spleen). The mean expression levels in the heart and brain were higher in comparison to the spleen 15 and 5.7 times, respectively. The mean expression in the kidney was only 1.7 times higher in comparison to the spleen. All differences in the mean *Pir* expression between the organs were statistically significant in a pair-wise fixed reallocation randomization test.[23]

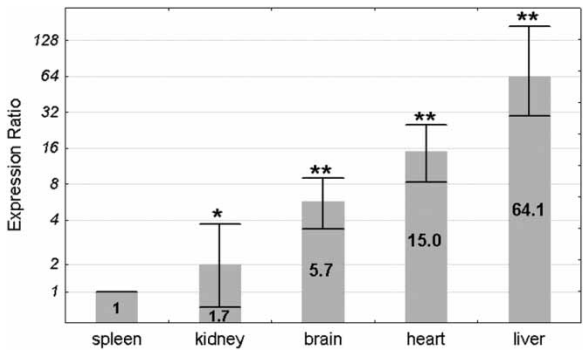

Figure 1 Mean *Pir* expression ratio in each organ of the wild-type mice. Expression ratios are presented relative to the spleen, where the expression level was the lowest and for clarity has been set as 1. Values are presented on a logarithmic scale. The exact expression ratio value for each organ is depicted on the respective column. Error bars show standard errors. Asterisks denote the statistical significance of the difference between each organ and the spleen (pair-wise fixed reallocation randomization test; **$P < 0.01$, *$P < 0.05$). $n = 16$ animals (8 males and 8 females).

We also compared the *Pir* expression in each organ between males and females, but we did not find any gender-related differences (data not shown).

Comparison of Pir expression between $Sod1^{+/+}$ *and* $Sod1^{-/-}$ *mice*

Mean *Pir* expression in each organ was compared between $Sod1^{+/+}$ and $Sod1^{-/-}$ mice. For each organ the expression in the $Sod1^{+/+}$ group was set as 1. The expression level in the $Sod1^{-/-}$ group was compared to the $Sod1^{+/+}$ by the ΔΔCt method and was shown as an expression ratio (fold change) over the $Sod1^{+/+}$ group. There were 16 animals in each group (8 males and 8 females). Significant increase in *Pir* expression in $Sod1^{-/-}$ mice was observed in the kidney and spleen. Mean expression in the kidney of Sod1 deficient mice was 2.54 times higher than in the wild-type animals. Similarly, the mean expression in the spleen of knock-out mice was 2.2 times higher than in the control group. In contrast to the spleen and kidney, there were no differences in *Pir* expression between $Sod1^{+/+}$ and $Sod1^{-/-}$ animals in the brain, heart, and liver (Fig. 2). Similar results were obtained when males and females were analyzed separately (Figs 3 and 4).

Discussion

Pirin expression in wild-type mice

The first report concerning pirin expression at mRNA level in different tissues was published by Wendler *et al.* in 1997.[1] It showed that the human *PIR* gene is differentially expressed in human tissues. Until now, there were no reports referring to the pirin expression pattern in other organisms. In the present paper, we showed that the mouse *Pir* gene transcription level varies significantly between organs in a way similar to its human ortholog. The highest

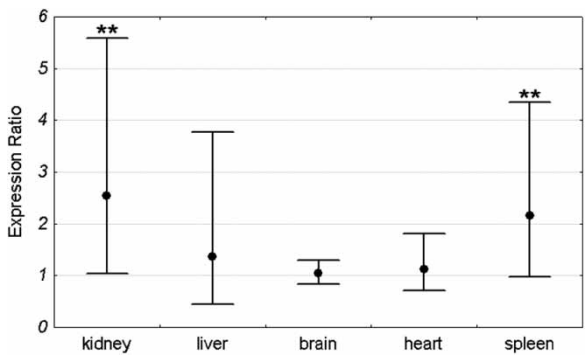

Figure 2 *Pir* expression ratio in each organ of $Sod1^{-/-}$ mice relative to wild-type animals. Dots represent mean expression ratios (fold changes) over the $Sod1^{+/+}$ and error bars represent standard errors. Asterisks denote the statistical significance of the difference between $Sod1^{-/-}$ and $Sod1^{+/+}$ (pair-wise fixed reallocation randomization test; **$P < 0.01$). $n = 16$ animals (8 males and 8 females) with each genotype.

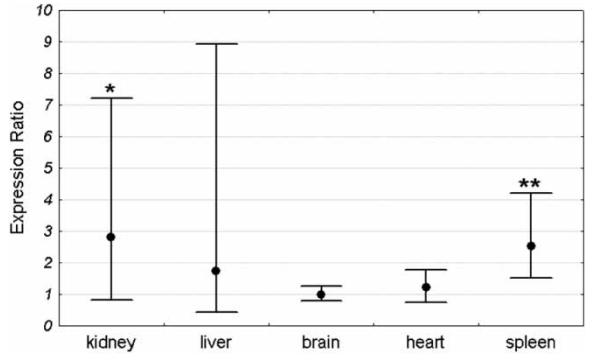

Figure 3 ***Pir*** **expression ratio in each organ of** ***Sod1***$^{-/-}$ **male mice relative to wild-type males. Dots represent the mean expression ratios (fold changes) over the** ***Sod1***$^{+/+}$ **and error bars represent the standard errors. Asterisks denote the statistical significance of difference between** ***Sod1***$^{-/-}$ **and** ***Sod1***$^{+/+}$ **(pair-wise fixed reallocation randomization test; **$P < 0.01$, *$P < 0.05$). $n = 8$ animals with each genotype.**

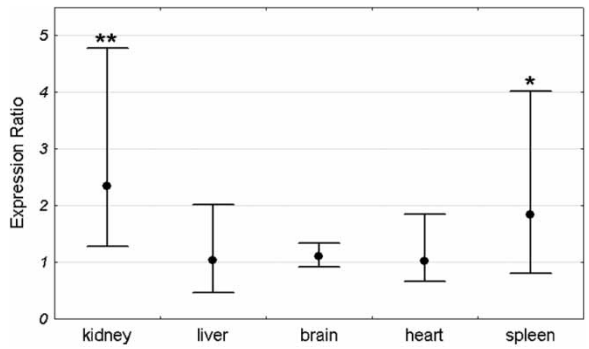

Figure 4 ***Pir*** **expression ratio in each organ of** ***Sod1***$^{-/-}$ **female mice relative to wild-type females. Dots represent the mean expression ratios (fold changes) over the** ***Sod1***$^{+/+}$ **and error bars represent the standard errors. Asterisks denote the statistical significance of difference between** ***Sod1***$^{-/-}$ **and** ***Sod1***$^{+/+}$ **(pair-wise fixed reallocation randomization test; **$P < 0.01$, *$P < 0.05$). $n = 8$ animals with each genotype.**

expression of both mouse and human pirin orthologs was found in the liver. However, whereas Wendler *et al.* reported that among all human tissues tested the lowest *PIR* mRNA levels were detected in the brain and pancreas; in our study, the lowest *Pir* expression was found in the mouse spleen and kidney. Wendler *et al.* did not study *PIR* expression in the human spleen and we did not determine *Pir* expression in the mouse pancreas, so we are not able to compare its expression in these organs between mice and humans. However, the comparison of the pirin transcription in the brain and kidney in both studies brought contradictory results. Whereas in humans the expression in the brain was clearly lower than in the kidney, in mice it was considerably higher, compared to the kidney. This result suggests that the pirin expression pattern can vary between species, but it also could have been an artifact due to different methods of mRNA quantification used in each study (northern blot vs. real-time PCR).

Pirin-coding genes both in mice and in humans are located on the X chromosome. This suggests that their expression may be gender related. Our results did not support this hypothesis, since we found no differences in *Pir* expression between males and females in any organ studied. This is probably due to the dosage-compensation mechanism, evolved to balance gene expression between males and females that in the case of mammals involve inactivation of one of the two X chromosomes in females.[24]

Differences in Pir expression between wild-type and Sod1-deficient mice

Previous reports suggesting pirin involvement in stress response and presenting its up-regulation under conditions of oxidative stress caused by cigarette smoke[5–7,12,13] encouraged us to test the hypothesis predicting that *Pir* expression is altered in Sod1-deficient mice. The experimental results supported the hypothesis, since among animals tested, mean *Pir* expression was significantly higher in the kidney and spleen of the *Sod1*$^{-/-}$ group, as compared to the *Sod1*$^{+/+}$ group. However, the effect of the Sod1 deficiency on *Pir* expression seems to be organ specific, as it was absent in the liver, heart, and brain. This organ-specific response may be related to differences in the level of *Pir* expression between organs. It seems meaningful that the effect was observed in the two organs which normally exhibit the lowest *Pir* expression. It seems plausible to assume that in other organs pirin level is high enough to meet the stress conditions arising from a Sod1 deficiency. It is worth noting that *Pir* expression in *Sod1*$^{-/-}$ mice increased to a similar extent both in males and females; considering *Pir* localization on the X chromosome, this was not an obvious result.

The detailed molecular mechanisms responsible for *Pir* up-regulation in *Sod1*$^{-/-}$ mice require further investigation, but the involvement of several transcription factors can be proposed: (1) as shown in this report, putative ARE elements are present not only in the human *PIR* gene but also in its mouse ortholog. The presence of ARE elements suggests a dependence on Nrf2 transcription factor that is activated in response to oxidative stress and up-regulates the expression of antioxidant genes to restore redox homeostasis. As already mentioned, pirin was identified as one of the Nrf2-modulated genes in response to cigarette smoke;[12] (2) Bergman *et al.*[25] reported over-expression of pirin in *c-JUN* transformed fibroblasts, an observation that strongly suggests the involvement of AP-1 transcription factor in the regulation of pirin expression. In our recent work, we have shown that *Fos* and *Jun* (both AP-1 subunits) expression was elevated in the kidney (*Fos* only) and liver (both *Fos* and *Jun*) of *Sod1*$^{-/-}$ mice.[26] It is

highly probable that changes in the expression of AP-1 subunits affect its activity, which in turn may have an influence on pirin expression; (3) it is also possible that *Pir* expression is related to NF-κB activity. It is well established that NF-κB activity is regulated by reactive oxygen species[27] and increase in NF-κB1 (p50) activity in kidney of *Sod1*$^{-/-}$ mice has recently been reported.[20] Since pirin forms complex with Bcl3 and p50,[2] the simultaneous increase in *Pir* expression and p50 activity in the same organ may not be merely a coincidence. However, we do not know whether it is the increased p50 activity that triggers up-regulation of *Pir* expression or conversely, whether the elevated pirin level is responsible for the increase in p50 activity. This needs further investigation.

In this work we have proved that the oxidative stress related gene *Pir* is significantly up-regulated in the mouse kidney and spleen under conditions of permanent oxidative stress resulting from Sod1 deficiency. Various results presenting pirin protein involvement in NF-κB signaling pathway regulation and its dependence on the activation of the stress-responsive transcription factor Nrf2 suggest that this protein may be classified as a putative proto-oncogene whose physiological activity certainly merits further studies.

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Conflict of interest statement: None declared.

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