Haemolytic anaemia and alterations in hepatic iron metabolism in aged mice lacking Cu,Zn-superoxide dismutase

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INTRODUCTION

ROS (reactive oxygen species) are by-products of normal aerobic metabolism. Several antioxidant systems exist in cells to provide protection against ROS toxicity. The principal defence against the toxicity of superoxide radical (O$_2^-$), released mainly into the cytosol [1], is SOD1 (superoxide dismutase 1; Cu,Zn-SOD). Surprisingly, despite this crucial protective role, homozygous mutant mice lacking Sod1 are viable and phenotypically normal [2–4].

Permanent oxidative stress occurring due to elevated ROS later in life is an important mechanism in the pathogenesis of aging [5]. In older mice, SOD1 deficiency results in an increased incidence of pathological changes, such as hepatocarcinogenesis, hearing loss and muscle atrophy [6–8]. As a consequence, the lifespan of Sod1-knockout mice is reduced by approx. 30 % [3,4].

Mice with genetic Sod1 deficiency have been extensively used as an experimental animal model to explore SOD1 dysfunction-related pathologies [6,8,9]. With regard to haematological disorders, the critical role of alterations in the redox state due to SOD1 deficiency has been reported in HA (haemolytic anaemia) occurring in Sod1−/− mice [10], as well as in the impairment of haematopoietic cell development in the mouse model of Fanconi anaemia [11].

In the present study we examined the consequences of HA in 1-year-old mice lacking Cu,Zn-SOD1 activity, focusing on alterations in their hepatic iron metabolism. Our results clearly indicate that the enhanced destruction and accelerated removal of RBCs (red blood cells) from the circulation of aged Sod1−/− mice induces substantial changes in the expression of genes involved in haem and iron homoeostasis. The results of the present study establish a new link between oxidative stress due to the combined effects of SOD1 deficiency and age-dependent changes in redox state and the regulation of iron metabolism at the systemic level.

EXPERIMENTAL

Animals

Mice homozygous for the non-functional Sod1 allele, and control mice homozygous for the wt (wild-type) Sod1 allele were used in experiments at the age of 1 year. A breeder pair of mice (strain B6;129S7-SOD1tm1Leb) heterozygous for a Sod1tm1Leb targeted mutation [3] were provided by The Jackson Laboratory. The Third Local Ethical Commission approved all experimental procedures involving animals.

Analysis of RBC and serum iron parameters

Blood was drawn from mice by direct cardiac puncture immediately after death and was treated with heparin. RBC and reticulocyte counts, and cell parameters, as well as serum iron, were determined using an automated Sysmex F-820 Analyser. Serum transferrin was measured at the Laboratory of Biochemistry at the Institut Federatif de Recherche 02, CHU Bichat-Claude Bernard (Paris, France).

Real-time quantitative RT (reverse transcriptase)–PCR

Hepatic Fpn (ferroportin), Hepc (hepcidin), CD91 and CD163 mRNA levels were measured by a real-time quantitative PCR
method as described previously [12]. Real-time quantitative RT–PCR of cDNAs derived from specific transcripts was performed in a Light Cycler (Roche Diagnostics) using the pairs of oligonucleotide primers indicated in Supplementary Table S1 (at http://www.BiochemJ.org/bj/420/bj4200383add.htm). For data analysis, Light Cycler 3.5 Software was used. Expression was quantified relative to that of a control transcript encoding GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Western blot analysis

For Western blot analysis, 50 μg of liver cytosolic extracts [for Ft (ferritin) subunit detection] or 100 μg of liver membrane extracts [for HO-1 (haem oxygenase-1) and Fpn detection] were resolved on SDS/PAGE (14% and 10% gels respectively). To determine plasma Hp (haptoglobin) and Hx (haemopexin) levels, samples were separated on SDS/PAGE (10% gels). Resolved proteins were electroblotted on to Hybond-ECL (where ECL is enhanced chemiluminescence) nitrocellulose membranes (Amersham Life Sciences). The membranes were initially blocked by gentle agitation in TBST (Tris buffered saline (50 mM Tris/HCl, pH 7.6, and 150 mM NaCl) containing 0.15% Tween 20) containing 5% fat-free skimmed milk powder for 1 h at room temperature (20°C) followed by overnight incubation at 4°C with the following primary antibodies: rabbit antiserum raised against recombinant mouse H- and L-Ft (heavy- and light-Ft respectively; kindly provided by Dr P. Santambrogio, Department of Bio Technology, San Raffaele Scientific Institute, Milan, Italy), a rabbit polyclonal antibody raised against rat liver HO-1 (StressGen), a rabbit antibody raised against mouse Fpn [13], a chicken antibody raised against human Hp (USBiological), and a goat antiserum raised against human Hx (kindly provided by Dr E. Tolosano, Molecular Biotechnology Center, University of Torino, Torino, Italy). Membranes were then washed and incubated with peroxidase-conjugated anti-rabbit, anti-chicken or anti-goat secondary antibodies (Santa Cruz Biotechnology) or 100 μg of liver cytosolic extracts [for Ft (ferritin) subunit detection] or 100 μg of liver membrane extracts [for HO-1 (haem oxygenase-1) and Fpn detection] were resolved on SDS/PAGE (14% and 10% gels respectively). Resolved proteins were electroblotted on to Hybond-ECL (where ECL is enhanced chemiluminescence) nitrocellulose membranes (Amersham Life Sciences). The membranes were initially blocked by gentle agitation in TBST (Tris buffered saline (50 mM Tris/HCl, pH 7.6, and 150 mM NaCl) containing 0.15% Tween 20) containing 5% fat-free skimmed milk powder for 1 h at room temperature (20°C) followed by overnight incubation at 4°C with the following primary antibodies: rabbit antiserum raised against recombinant mouse H- and L-Ft (heavy- and light-Ft respectively; kindly provided by Dr P. Santambrogio, Department of Bio Technology, San Raffaele Scientific Institute, Milan, Italy), a rabbit polyclonal antibody raised against rat liver HO-1 (StressGen), a rabbit antibody raised against mouse Fpn [13], a chicken antibody raised against human Hp (USBiological), and a goat antiserum raised against human Hx (kindly provided by Dr E. Tolosano, Molecular Biotechnology Center, University of Torino, Torino, Italy). Membranes were then washed and incubated with peroxidase-conjugated anti-rabbit, anti-chicken or anti-goat secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactive bands were detected using the ECL Plus Western blotting detection system (Amersham Life Sciences). Quantification was performed relative to actin detected using a specific antibody (Santa Cruz Biotechnology).

Immunohistochemical staining

Mouse livers were fixed in Bouin’s fixative for 72 h at 20°C and then embedded in paraffin for sectioning. After mounting on glass slides, 5 μm sections were processed for immunofluorescence. The sections were first counterstained with 0.1% Methylene Blue in PBS and mounted in Permount. The sections were then incubated in a humid chamber at room temperature for 16 h using the following dilutions in blocking solution: anti-Fpn, 1:75 and anti-HO-1, 1:100. After three washes with PBS/0.5% BSA, the sections were incubated for 1 h at room temperature with a goat anti-rabbit Alexa Fluor® 488-conjugated secondary antibody (Molecular Probes) diluted 1:200 in blocking solution. After washing, the slides were mounted on cover slips with anti-fading mounting reagent (Prolong Antifade kit P-7481, Molecular Probes) and examined for immunofluorescence.

Quantitative hepatic non-haem iron measurement and hepatic iron staining

Hepatic non-haem iron content was determined by acid digestion of liver samples at 65°C for 20 h, followed by colorimetric measurement of the absorbance of the iron–ferrozine complex at 560 nm as described previously [14]. Deparaffinized liver sections were stained with Prussian Blue and counterstained with Nuclear Red using standard procedures.

FACS analysis of PS (phosphatidylserine) externalization

The presence of PS on the surface of mouse RBC membranes was assessed using Annexin V–FITC staining (Calbiochem) following the manufacturer’s protocol. Stained cells were analysed by flow-cytometry (FACS Calibur), and data were processed using CellQuest software (Becton Dickinson).

EP (erythrophagocytosis) assay

The EP assay was performed as described previously [15] using BMDMs (bone marrow-derived macrophages) isolated from the femurs of Sod1−/− mice and fresh RBCs obtained from 1-year-old mice of both Sod1−/− and Sod1+/+ genotypes.

Haem content measurement

The haem content of formic acid-solubilized BMDMs was determined spectrophotometrically at 398 nm using haemin standards prepared in formic acid and a molar absorption coefficient of 1.5 × 104 M−1 · cm−1 [16].

Statistical evaluation

Where suitable, one-way ANOVA was applied to the data. The Scheffe test was used to estimate the significant difference (P < 0.05) between means. All calculations were performed using Statgraphics Plus 6.0 software.

Results

Moderate regenerative anaemia in aged Sod1−/− mice

One-year-old Sod1−/− mice show moderate anaemia with reductions of approx. 25% in both erythrocyte number and Hb concentration, and a reduction of approx. 14% in the haematocrit value (Table 1). Values for the mean cell Hb concentration were unchanged, which, together with normal levels of serum iron (Figure 1A) and transferrin (Figure 1B), may suggest that the anaemia in Sod1−/− animals is not associated with iron deficiency. The raised number of peripheral reticulocytes in Sod1−/− mice (Figure 1C), as well as the increased spleen index values (Figure 1D) indicate that the anaemia in Sod1−/− mice is regenerative.

Hb and haem scavenging systems in Sod1−/− mice

The findings obtained by Iuchi et al. [10] indicate that Sod1−/− RBCs are prone to haemolysis due to oxidative damage. Therefore we attempted first to identify hallmarks of intravascular haemolysis. We measured plasma levels of Hp and Hx, two glycoproteins involved in the clearance of Hb and haem respectively, from the bloodstream. When Hb and haem are released...
Serum iron (A) and serum transferrin (B) levels in Sod1−/− and Sod1+/+ mice. n = 8–10 for each genotype. (C) Increased number of reticulocytes in peripheral blood of Sod1−/− mice. *P < 0.05, significant differences between values for Sod1−/− and Sod1+/+ mice. (D) Increased spleen index in Sod1−/− mice. Spleen index was calculated according to the formula: spleen index = \( \sqrt{\text{spleen weight/body weight}} \) [45].

from ruptured erythrocytes they are instantly bound by Hp and Hx and form Hp–Hb and Hx–haem high-affinity complexes [17]. These complexes are then rapidly taken up from the circulation by CD163 [18] and CD91 [19] receptors present on tissue macrophages and hepatocytes. The rationale for measuring Hp and Hx in the plasma is that CD163 and CD91 receptors have no measurable affinity for free Hp or Hx. Thus specific recognition of Hp–Hb and Hx–haem complexes by CD163 and CD91 explains the decrease in Hp or Hx concentration in the plasma during accelerated haemolysis [19,20]. Indeed, we found that the Hp level in plasma is strongly decreased in Sod1−/− mice (Figure 2A). Surprisingly, higher plasma concentrations of Hx were detected in these animals compared with wt littermates. Considering the divergent patterns of Hp and Hx protein abundance in the plasma of Sod1-deficient mice, we analysed the expression of Hpx and Hps (the mouse Hp and Hx genes respectively) genes in the liver, the major site of Hp and Hx synthesis [17]. The results shown in Figure 2(B) indicate that Hp, as well as Hx, mRNAs are markedly up-regulated. We also measured expression of CD163 and CD91 transcripts in the liver, coding for receptors of Hp–Hb and Hx–haem complexes respectively [18,19]. Levels of CD163 mRNA showed no differences between Sod1−/− and Sod1+/+ mice, but the abundance of CD91 mRNA was significantly lower in Sod1-deficient mice (Figure 2C).

**Indices of extravascular haemolysis in Sod1-deficient mice**

To investigate the susceptibility of RBCs to be removed from the circulation by EP, as a potential mechanism underlying HA, we evaluated the presence of PS externalized on the membranes of circulating RBCs. Flow-cytometric detection of externalized PS clearly showed a more than 3-fold increase in the percentage of PS-positive Sod1−/− RBCs when compared with wt RBCs (Figure 3A). In artificially aged RBCs [15], the proportion of PS translocated to the cell surface was significantly greater than the values found for Sod1−/− and Sod1+/+ RBCs. We next used an EP assay to examine the correlation between the levels of externalized PS with the haem content in Sod1−/− BMDMs after phagocytosis of RBCs of the two genotypes. As expected, the haem content in BMDMs after incubation with Sod1−/− RBCs was significantly greater than after incubation with wt RBCs (Figure 3B) indicating that Sod1−/− RBCs are more readily phagocytosed than Sod1+/+ RBCs.

**Increase in hepatic HO-1 expression in Sod1−/− mice**

To better understand the role of HO-1 during accelerated haemolysis, we analysed its expression in the liver, at both the mRNA and protein levels (Figure 4). Levels of HO-1 mRNA were increased approx. 3-fold in Sod1−/− mice compared with wt littermates (Figure 4A), and HO-1 protein levels assessed by both Western blot analysis (Figure 4B) and immunofluorescence (Figure 4C) were similarly elevated. Interestingly, HO-1 expression in liver was mostly detected in Sod1−/− KCs (Kupffer cells) in accordance with its known high expression in phagocytes. In contrast with wt mice, Hb autofluorescence was strongly present in liver sections from anaemic Sod1−/− mice (Figure 4D). Interestingly,
indices of increased extravascular haemolysis in Sod1-deficient mice

(A) Increased externalization of PS on Sod1+/− RBCs. Exposure of PS was assessed by staining cells with Annexin V–FITC followed by flow cytometry. Artificially aged Ca2+ ionophore A23187-treated RBCs from Sod1+/− mice were used as control cells showing high levels of PS exposure on their cell membrane. Stained RBCs as a percentage of the total number of RBCs was calculated. Results are shown as the means ± S.D. for three wt mice and four Sod1+/− mice. Significant differences in PS exposure are indicated. (B) Increased haem content of BMDM after in vitro phagocytosis of erythrocytes. The EP assay was performed using BMDDMs and RBCs from 1-year-old Sod1+/− and Sod1+/+ mice. Haem content of the formic acid-solubilized BMDDMs was determined spectrophotometrically at 398 nm. Values represent the means ± S.D. of four EP assays performed in duplicate on RBCs from four mice of each genotype. Significant differences in PS exposure are indicated (P < 0.05).

Hepatic iron status in haemolytic Sod1+/− mice

Massive iron accumulation in tissues is one of the characteristic metabolic features of acute HAs of various aetiologies [21]. We therefore examined whether this iron overload phenotype could be observed in haemolytic Sod1+/− mice. Staining of non-haem iron in the liver showed iron accumulation in some Sod1+/− liver macrophages (Figure 5A). On the other hand, there was no evidence of global iron loading in hepatocytes. Furthermore, we observed no significant change in non-haem iron content in the liver (Figure 5B). Accordingly, a significant decrease in L-Ft protein (Figure 5C) in hepatic cytosolic extracts from Sod1+/− mice was observed. The protein level of H-Ft showed no difference between Sod1+/+ and Sod1+/− mice.

Hepatic Hepc mRNA and Fpn protein levels are decreased and increased respectively in Sod1-deficient mice

We next studied the expression of Fpn, the only known iron exporter in mammalian cells. Western blotting of hepatic membrane extracts (Figure 6A) and immunofluorescence on histological liver sections (Figure 6B), showed that Fpn is expressed at low levels in the livers of Sod1+/+ mice. In contrast, Fpn expression was markedly enhanced in the livers of Sod1+/− mice (Figures 6A and 6B). Microscopic analysis of immunofluorescence staining on liver sections (Figure 6B) identified KCs as the principal cell type of Fpn expression. Enlargement of immunofluorescent pictures also indicated the presence of RBC Hb autofluorescence in Sod1+/− KCs strongly positive for Fpn (see the enlargement in Figure 6B). At the level of mRNA, we detected a slight, but not significant, change in Fpn mRNA expression between Sod1+/+ and Sod1+/− animals (Figure 6C). Considering the established role of Hepc in the degradation of Fpn molecules [22], we then analysed Hepc mRNA expression in mice of both genotypes and found that, in Sod1+/− mice, the expression of Hepc mRNA was decreased by approx. 70% (Figure 6D).

DISCUSSION

In the present study, we have investigated the consequences of oxidative stress due to the combined effects of SOD1 deficiency [4,6,10] and age-dependent alterations in redox state [4,6] on iron homoeostasis in aged mice. Considering the well-known susceptibility of RBCs to oxidative stress, and the importance of the liver in recycling haem iron, we focused the present study on the peripheral erythrocyte–liver axis. Furthermore, analysis of markers of haem-iron recycling (HO-1, Fpn) and of the macrophage phagosome maturation (Lamp1 [lysosome-associated membrane protein 1]) in the spleen of Sod1+/− mice indicates no major changes in haem-iron recycling in this tissue...
Our first observation concerning 1-year-old mice lacking Cu,Zn-SOD activity was a reduction in the values of the main RBC parameters. A similar level of anaemia has been reported in 40-week-old Sod1−/− mice [10]. It has been proposed that oxidative stress is the major factor causing SOD1-deficiency anaemia to qualify as a HA [10]. Because the mechanisms underlying HA are believed to rely on the destruction or accelerated removal of RBCs from the circulation, we looked for evidence of intra- and extra-vascular haemolysis in aged Sod1−/− mice.

Hb and haem are the most abundant iron compounds released into the plasma from ruptured RBCs and they have been implicated in toxic effects [23]. Hp and Hx, being scavengers of Hb and haem respectively, are considered plasma-protective proteins against intravascular haemolysis [17]. The increased hepatic Hp gene expression and reduced plasma Hp protein level we observed in Sod1−/− mice may reflect the induction of Hp synthesis in the liver, its release into the circulation followed by formation of the Hp–Hb complex, and its clearance by KCs via CD163 [18]. Accordingly, the plasma level of Hp is usually reduced in haemolytic states [17]. With regard to Hx, the increased levels found in the plasma of Sod1−/− mice could be due to the fact that the amount consumed by moderate haemolysis is outweighed by its enhanced production as a result of inflammation in the liver. Furthermore, the plasma Hx level starts to decrease when the Hb-binding capacity of Hp is exceeded [24]. Finally, low hepatic expression of CD91 mRNA (suggesting low protein expression) may also explain the high levels of Hx detected in the plasma of Sod1−/− mice. In support of a role for enhanced extravascular haemolysis, i.e. accelerated EP, in HA in mice lacking SOD1, we observed the presence of RBC Hb autofluorescence in liver macrophages attesting to the high EP activity of these cells, although the Hb autofluorescence signal may also derive from internalized Hp–Hb complexes. We also demonstrated marked externalization of PS on the surface of Hp synthesis in the liver, its release into the circulation followed by formation of the Hp–Hb complex, and its clearance by KCs via CD163 [18]. Accordingly, the plasma level of Hp is usually reduced in haemolytic states [17]. With regard to Hx, the increased levels found in the plasma of Sod1−/− mice could be due to the fact that the amount consumed by moderate haemolysis is outweighed by its enhanced production as a result of inflammation in the liver. Furthermore, the plasma Hx level starts to decrease when the Hb-binding capacity of Hp is exceeded [24]. Finally, low hepatic expression of CD91 mRNA (suggesting low protein expression) may also explain the high levels of Hx detected in the plasma of Sod1−/− mice. In support of a role for enhanced extravascular haemolysis, i.e. accelerated EP, in HA in mice lacking SOD1, we observed the presence of RBC Hb autofluorescence in liver macrophages attesting to the high EP activity of these cells, although the Hb autofluorescence signal may also derive from internalized Hp–Hb complexes. We also demonstrated marked externalization of PS on the surface
of RBCs from Sod1<sup>−/−</sup> mice that probably contribute to this observation. Indeed, under physiological conditions the exposure of PS on the RBC membrane is a signal for macrophages to recognize damaged or aged RBCs [25]. A role for extravascular haemolysis in the mechanism contributing to HA is further supported <i>ex vivo</i> by the high haem content in BMDMs after incubation with RBCs from Sod1<sup>−/−</sup> mice. This observation clearly indicated the increased susceptibility of Sod1<sup>−/−</sup> RBCs to be engulfed by phagocytes.

In the present study, we characterize large changes in hepatic iron metabolism in aged Sod1-deficient mice displaying mild, but chronic, HA. We first analysed the expression of HO-1, an enzyme responsible for haem catabolism in macrophages [26]. We found a substantial increase in HO-1 mRNA and protein expression in the liver of Sod1<sup>−/−</sup> mice in comparison with the wt mice. In addition, HO-1 was found to be localized in KCs, which exhibited strong Hb fluorescence from ingested RBCs. In accordance with our results, increased hepatic HO-1 expression has also been reported in various haemolytic states [27,28], and in macrophages in an EP assay [15]. The role of HO-1 in systemic iron turnover has been confirmed in HO-1 deficiency [29,30]. The anaemia and hepatic iron overload observed in HO-1-deficient subjects demonstrate the importance of this enzyme in recycling haem iron. On the other hand, it has been reported that potentiated HO-1 expression does not counteract tissue iron loading in acute HA [28,31]. We found that the moderate HA occurring in Sod1<sup>−/−</sup> mice is associated with hepatic deposits of non-haem iron in KCs. Therefore the increased expression of HO-1 in Sod1<sup>−/−</sup> liver macrophages probably participates in the iron release from haem into the cytosol and its subsequent storage into Ft deposits observed in these cells. We also observed a strong up-regulation of the iron exporter Fpn in KCs that have ingested RBCs. Indeed, Fpn is highly expressed in tissue macrophages, including KCs [32–34]. Hepc, a peptide secreted by the liver, binds to Fpn and causes its degradation [22]. We found that Hepc levels were reduced in Sod1<sup>−/−</sup> mice. It has been proposed that the occurrence of elevated erythropoiesis is sufficient to explain the decreased expression of Hepc by an as yet unidentified erythroid regulator negatively controlling Hepc synthesis [35]. Indeed, enhanced erythropoietic activity as a compensatory reaction in HA in Sod1<sup>−/−</sup> mice has been demonstrated by means of increased reticulocyte counts and splenomegaly (the present study, [10]). Interestingly, expansion of
through efficient clearance of these iron compounds from the oxidative stress due to SOD1 deficiency and age-related decreases in haem metabolism triggered in response to modest HA induced by Fpn protein of Sod1L-Ft, mostly expressed in these cells [42], tends to decrease in the liver is well-documented [38–40], its relevance was associated with increase in the export of iron after EP [41]. Interestingly, no iron deposit was found in hepatocytes, and L-Ft, mostly expressed in these cells [42], tends to decrease in Sod1L-/- liver. However, observations indicate that decrease of hepatic L-Ft in Sod1-deficient mice reflects hepatic secretion in response to inflammation [43] rather than iron depletion in these cells via up-regulation of Fpn. Despite clear mRNA expression of the Fpn1 gene in hepatocytes [44], the protein expression and its subcellular localization in these cells remain poorly documented [39]. Furthermore, in mice lacking the Hamp (hepcidin antimicrobial peptide) gene, an up-regulation of Fpn in liver did not prevent iron overload in hepatocytes, whereas KCs were iron deficient [39,45]. Finally, in contrast with KCs, we did not detect any specific labelling of Fpn in Sod1L-/- hepatocytes.

In summary, we have characterized large changes in iron and haem metabolism triggered in response to modest HA induced by oxidative stress due to SOD1 deficiency and age-related decreases in antioxidant defence. The changes act to reduce the toxicity of Hb and/or haem released from oxidatively damaged RBCs through efficient clearance of these iron compounds from the circulation. In addition, combined up-regulation of HO-1 and Fpn in KCs of Sod1L-/- mice suggest that at least a part of haem iron is recycled into the circulation probably to respond to the needs of erythropoiesis.

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SOD1 deficiency, haemolytic anaemia and iron metabolism 389

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SUPPLEMENTARY ONLINE DATA

Haemolytic anaemia and alterations in hepatic iron metabolism in aged mice lacking Cu,Zn-superoxide dismutase

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Figure S1 Expression of Fpn, HO-1 and Lamp1 in the spleen of 1-year-old knockout Sod1 and wt mice

(A) HO-1 and Fpn levels in spleen membrane protein extracts as assessed by Western blotting with specific antibodies. (B) Lysosome-associated membrane protein 1 (Lamp1, a marker of macrophage phagosome maturation) and vinculin levels in spleen total protein extracts as assessed by Western blotting with specific antibodies (obtained from Santa Cruz Biotechnology and Invitrogen respectively).

Table 1 Oligonucleotide primers used for real-time quantitative RT–PCR analysis

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