Disruption of paraoxonase 3 impairs proliferation and antioxidant defenses in human A549 cells and causes embryonic lethality in mice

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Kempster SL, Belteki G, Licence D, Charnock-Jones DS, Smith GC. Disruption of paraoxonase 3 impairs proliferation and antioxidant defenses in human A549 cells and causes embryonic lethality in mice. Am J Physiol Endocrinol Metab 302: E103–E107, 2012. First published September 27, 2011; doi:10.1152/ajpendo.00357.2011.—We had shown previously that paraoxonase 3 (PON3), a putative circulating antioxidant, was systemically upregulated in late-gestation rat, sheep, and human fetuses. Our overarching hypothesis is that preterm human infants are delivered with low levels of PON3 and that this contributes to a state of oxidative stress. We sought to determine whether absence of PON3 was associated with reduced neonatal viability in mice and studied the offspring from crosses between Pon3−/− and wild-type littermates at E10.5 and E17.5.

METHODS

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Prevents transcription further downstream. Correct gene targeting was confirmed by Southern blot using 5′ and 3′ probes outside the homology arms by the Texas A & M Institute for Genomic Medicine. To determine the effect of Pon3 loss on fetal development, embryos were collected at embryonic day (E)10.5 and E17.5 from heterozygous timed matings, where day of plug = E0.5. To determine the effect of Pon3 loss in neonatal mice, pups were euthanized on the morning following delivery (P1; delivery occurs during the night). Genomic DNA was isolated by phenol chloroform extraction and ethanol precipitation and used as a template for genotyping by PCR with a Pon3-specific reverse primer (5′-AAGGCTGAGTCAAGCCTGTCGC-3′) and forward primers directed against either Pon3 or the inserted neoeym cassette (5′-TTCTTTAGGCACTGTGGATTAC-3′ and 5′-GCAGCCGATCGCCTTCTATC-3′, respectively). PCR with Taq polymerase (Bioline, London, UK) conditions consisted of denaturation at 95°C for 5 min and 40 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 1 min, followed by a final extension of 72°C for 10 min in the presence of 3 mM MgCl2 and 0.5 mM primers.

**Detection of Pon3 mRNA in mouse tissue.** Total RNA was extracted from E17.5 mouse embryonic lungs using Trizol (Invitrogen, Paisley, UK) according to the manufacturer's instructions. DNAse treated (Promega, Southampton, UK), and reverse transcribed to cDNA with Superscript II (Invitrogen) with random hexamer primers. cDNA was used as template for PCR with Pon3- and β-actin-specific primers. A 362-bp fragment relating to mouse Pon3 was amplified with primers against the 3′ end of Pon3 mRNA (5′-GATCTTTGACCTT-CACTTGA-3′ and 5′-TTTGTCAATAAGCGCAAGG-3′), with PCR cycling conditions of 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final extension of 72°C for 10 min. A 527-bp β-actin PCR product was amplified using β-actin-specific forward and reverse primers (5′-CTAACATGAGTTGGCATGTG-3′ and 5′-GCTCTTCTTCCAGCCTTCTT-3′). PCR cycling conditions included an initial denaturation of 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 48°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min.

**Studies of human cell lines.** The human lung adenocarcinoma cell line A549 was employed to study the effects of Pon3 knockdown. Cells were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, and antibiotics [penicillin (50 U/ml) and streptomycin (50 μg/ml)]. Permanent knockdown of Pon3 was achieved using Sure-Silencing short hairpin RNA (shRNA) plasmids purchased from SA Biosciences (Frederick, MD), and all experiments were conducted in the presence of 21% oxygen. We employed four different short hairpins targeting Pon3 and a nontargeting shRNA. To generate A549 cell lines stably expressing shRNAs, 2 × 105 cells/well were plated on 24-well plates in medium with no antibiotics. Cells were transfected 24 h later using 1 μg of supercoiled plasmid, 2 μl of Lipofectamine 2000, and 150 μl of Optimem. The next day, cells were passaged onto 3.5-cm dishes, and transfected cells were selected with G418 (400 μg/ml) for 21 days. The resultant colonies were trypsinized to generate a polyclonal population of cells expressing the shRNA plasmids. Cell proliferation was assessed by Thiazolyl Blue Tetrazolium Bromide (MTT) assay, as described previously (13); 10,000 cells/well were plated onto 96-well plates (n = 16 for each cell line). Cells were grown in G418-containing medium (400 μg/ml) for 72 h. Twenty microliters of MTT (5 mg/ml solution) was added to each well, and the cells were incubated at 37°C for 1 h. Medium was removed and 100 μl of DMSO added. Optical density was determined at 550 and 690 nm.

Transient knockdown of Pon3 was achieved using short interfering RNAs (siRNAs). 1.5 × 10^5 A549 cells were plated onto each well of a six-well plate (35-mm diameter) in medium without antibiotics and cultured for 72 h at 37°C in an atmosphere of 2% O2 and 5% CO2 in a Biospherix Xvivo controlled atmosphere tissue culture system (Biospherix, Lacona, NY). Cells were then transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In brief, 100 pmol of siRNA (against Pon3 and nontargeting siRNA, pool of 4, On-Target Plus; Dharmacon) was used along with 1 μl of Lipofectamine 2000 and 500 μl Opti-Mem Reduced Serum Medium (Invitrogen) per well. Eight transfections were done with both Pon3 siRNA and nontargeting siRNA. All biological replicates were transfected individually. Transfection was performed overnight in 2% O2. After 24 h, cells were passaged 1:6 using reagents equilibrated at 2% oxygen and plated onto six-well plates in medium with antibiotics. Each six-well plate contained three wells from one of the transfections with siPon3 and three wells from one of the transfections with nontargeting siRNA. Forty-eight hours after the transfection, plates were placed in incubators with low (2%) or ambient (21%) oxygen. After a further 24 h (i.e., 72 h after the start of the transfection), cells were harvested using trypsin (for RNA analysis) or with a cell scraper for antioxidant activity assay. Cells for RT-PCR analysis were resuspended in 350 μl of RA1 buffer supplemented with β-mercaptoethanol (Nucleospin RNA II; Macherey-Nagel, Düren, Germany), frozen immediately in dry ice, and stored at −80°C. Scraped cells for antioxidant activity assay were stored at −80°C. All analyses were of transfected and control cells processed in parallel over the same period of 72 h.

**Quantitative real-time RT-PCR of A549 cells.** Total RNA was purified from cells using Nucleospin RNA II kit (Macherey-Nagel) with on-column DNAse treatment. Total RNA was reverse transcribed to cDNA using superscript II (Invitrogen) with random hexamer primers and used as template for quantitative RT-PCR. PCR was performed with an ABI Prism 7900HT system (Applied Biosystems, Warrington, UK) with Fast QPCR mix (Applied Biosystems) and primers and probes for PON3, GAPDH, and 18S RNA. These primers and probes were pre-designed and preoptimized (Hs00412993_m1, Hs02758991_g1, and Hs99999901_s1, respectively; Applied Biosystems). All samples were assayed in triplicate. PON3 transcript levels were quantified using normalization to the geometric mean of GAPDH and 18S RNA (19).

**Western blotting.** Samples (mouse lung, liver, or A549 cells) were homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl2, 2 mM EDTA, 1% Nonidet P40, and 0.1% SDS) with protease inhibitors (Roche). Fifty micrograms of protein per lane was reduced by addition of Nupage Sample Reducing agent (Invitrogen), heat-denatured, and loaded on a 10% Novex Bis-Tris Gel (Invitrogen). After electrophoresis, proteins were transferred to a PVDF membrane (Invitrogen), blocked with 5% skimmed milk in TBS-Tween 20 (0.05%) for 1 h, and subsequently probed with a goat polyclonal anti-human PON3 antibody (Lifespan Biosciences) at 0.2 μg/ml (0.05%) for 1 h, and then probed with a goat polyclonal antibody to human GAPDH (Abcam) at 0.2 μg/ml to confirm equal loading of protein.

**Analysis of total antioxidant capacity.** Total antioxidant capacity was measured using a commercial antioxidant assay kit (Cayman Chemicals; Cambridge Bioscience, Cambridge, UK). This assay estimates the sum of all antioxidant activity in a sample by quantifying its ability to prevent oxidation of 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] by metmyoglobin relative to Trolox (a water-soluble tocopherol analog). Briefly, cell pellets were resuspended in lysis buffer (5 mM potassium phosphate, pH 7.4, 0.9% NaCl, and 0.1% glucose) and centrifuged. The supernatant was analyzed for antioxidant capacity using 10 μl of sample, 10 μl of metmyoglobin, and 150 μl of chromogen added to the well of a 96-well plate, and all samples were assayed in duplicate. Forty microliters of H2O2 was added to each well and the plate incubated at room temperature for 5 min. Absorbance was read at 750 nm. Antioxidant capacity was expressed in Trolox equivalents using a standard curve generated by serial dilution of a standard Trolox solution.
**RESULTS**

Pon3 knockout mice showed no Pon3 mRNA in liver or lung at E17.5 by RT-PCR, whereas it was clearly present in heterozygous and wild-type littermates (Fig. 1A). Uniformity of cDNA synthesis was established by RT-PCR for the housekeeping gene β-actin. Western blotting also showed no Pon3 protein in either lung or liver from knockout mice but confirmed the presence of Pon3 protein in wild-type and heterozygous litters of the expected size (40 kDa). The membrane was stripped and reprobed for GAPDH, which confirmed equal loading of protein (data not shown). When the offspring of het/het matings were studied, there were four null mutants out of 43 offspring from eight litters [9.3%, 95% confidence interval (CI) = 2.6–22.1%] at E10.5 and five null mutants out of 63 offspring from seven litters (7.9%, 95% CI = 2.6–17.6%) at E17.5; the proportions were significantly less than the expected 25% at both gestational ages (P < 0.001). On the first day of postnatal life (P1), there were three null mutants out of 127 offspring from 23 litters (2.4%, 95% CI = 0.5–6.7%), which was significantly less (P = 0.04) than the proportion observed in fetal life. It is likely that dead neonates were ingested by the mothers. After null mutants were excluded, 25 out of 39 fetuses at E10.5 were heterozygous (64.1%, 95% CI = 47.2–78.8), 33 out of 58 fetuses at E17.5 were heterozygous (56.9%, 95% CI = 43.2–69.8), and 81 out of 124 neonates at P1 were heterozygous (65.3%, 95% CI = 56.3–73.6). In all cases, the 95% CIs included the 66.7% heterozygotes predicted by Mendelian ratio (with null mutants having been excluded).

The effect of Pon3 heterozygosity on fetal growth was studied by mating six heterozygous animals with six wild-type animals. The placental and body weight of Pon3 heterozygous fetuses at E17.5 was 94.1% (95% CI = 91.0–97.1, P < 0.001) of their wild-type littermates (n = 24). The reduction in body weight was similar whether the Pon3 mutant allele was inherited from the mother (96.0%, 95% CI = 93.1–98.9, 3 litters of 22 pups, 12 heterozygous) or father (92.3%, 95% CI = 86.8–97.8, 3 litters of 27 pups, 13 heterozygous). The placental weight was 85.7% of the mean of the wild-type littermates, which was reduced significantly (95% CI = 81.2–90.2, P < 0.001). The placental weight was slightly smaller when the mutant allele was paternally inherited (81.6%, 95% CI = 76.2–87.1 vs. 86.7%; 95% CI = 81.8–93.3 for maternal inheritance), but this difference was not statistically significant (P > 0.05).

We next conducted a series of experiments using a human cell line, A549, derived from human lung adenocarcinoma. Using two different shRNAs, we permanently reduced PON3 expression in these cells. Compared with cells expressing a nontargeting shRNA, PON3 transcript levels were reduced by an average of 43 and 55% resulting in a 62 and 55%, reduction, respectively, in PON3 protein level. Both knockdown cell lines demonstrated significantly reduced cell proliferation in 21% oxygen using MTT assay compared with control cells expressing the nontargeting shRNA (61.7 and 58.2% expression, 95% CI = 52.4–71.0 and 51.7–64.6, respectively; n = 16 for each group, P < 0.001). We next studied transient knockdown of PON3 using siRNA in 2% oxygen, and after 24 h cells were either exposed to 21% oxygen for 48 h or maintained in 2% oxygen. At the end of this (72 h after transfection), total antioxidant capacity and PON3 transcript levels were compared. PON3 transcript levels were reduced significantly in both 2 and 21% oxygen (average reduction 64 and 68%, respectively). PON3 knockdown had no effect on total antioxidant capacity in 2% oxygen but was associated with significantly reduced total antioxidant capacity in 21% oxygen (P = 0.0078; Fig. 2).

**DISCUSSION**

The key finding of the current study is that the absence of PON3 had adverse effects both on mice and in a human cell line. When studying the offspring of matings of animals that were heterozygous for null mutation of the Pon3 gene, we found that the proportion of homozygous null mutants was well below the expected Mendelian ratio at E10.5. There was no further significant decline in the proportion of null mutants between E10.5 and E17.5. However, there was a further significant reduction in the proportion of null mutants on neonatal day 1. On the basis of our previous studies, we had hypothesized that upregulation of PON3 was an important preparative process for birth and that absence of PON3 may be harmful following birth. The increased rate of neonatal loss is clearly supportive of that hypothesis. However, there was no direct evidence, to our knowledge, that PON3 protected cells from the effects of increased levels of oxygen. To address this, we studied a human cell line (A549). First, we demonstrated that stable knockdown of PON3 in ambient oxygen was associated with reduced cell proliferation. However, such an effect could be through multiple mechanisms other than through oxidative stress. Hence, we next sought to determine the effects of the absence of PON3 (using siRNA knockdown) on total antioxidant capacity in low (2%) and ambient (21%) oxygen. We...
found that PON3 knockdown had no effect on total antioxidant capacity in low oxygen but resulted in reduced total antioxidant capacity in ambient oxygen. We conclude that Pon3 has a nonredundant biological role in both early murine development and adaptation to extrauterine life. Moreover, we provide direct evidence that PON3 acts as an antioxidant in the presence of atmospheric oxygen levels in human cells.

The motivation for studying the Pon3 knockout was to determine whether absence of Pon3 was associated with increased neonatal mortality. However, when comparing the number of null mutants in fetal life, we found that the proportion was well below the predicted 25% Mendelian ratio. This was the case at both E10.5 and E17.5, and there was no difference in the number of reabsorbed fetuses at these time points. These observations suggest that there was loss of Pon3 null mutants in the first half of pregnancy and, therefore, that Pon3 must have an important nonredundant role in early development. Further studies will be required to determine the mechanism of these early losses. Previous studies have demonstrated that Pon3 mRNA is present in preimplantation embryos [see GEO data series GDS578 from Hamatani et al. (6)] and that Pon3 is expressed in early embryonic life (10). It is possible that increased rates of early loss may be explained by the absence of the antioxidant effect of PON3. However, in addition to their role as antioxidants, paraoxonases are capable of metabolizing derivatives of important regulatory hormones, have antiatherogenic and anti-inflammatory properties, can detoxify or degrade xenobiotics, and have the potential to alter cell or tissue type that determines the early lethality of null mutants. However, we observed that animals that were heterozygous for the Pon3 null allele exhibited a phenotype; namely, the body weight and placental weight were lower than wild-type littermates at E17.5, and we considered that this may be due to imprinting. Hence, we studied body and placental weight in the mating of heterozygous mothers and wild-type fathers. Body weight and placental weight were similar among wild-type fathers and wild-type mothers and heterozygous fathers. Body weight and placental weight were similar among the heterozygotes irrespective of the parent of origin of the mutant allele, which is not supportive of imprinting.

An analysis of a number of genes on mouse chromosome 6 demonstrated that Pon3 expression in the placenta and embryo was maternally biased at E10 and that the degree of maternal bias was greater in the placenta. However, these authors commented that because the C57Bl6 allele was always expressed more strongly, the maternal/paternal ratios observed were not a good indicator of imprinting in this case (10). If PON3 was strongly imprinted, we would have expected a reduction in the normal 2:1 ratio of heterozygotes to wild types following matings of pairs of heterozygous animals. Given the normal ratio of heterozygotes, we conclude that the gene is not expressed exclusively from the maternal or paternal allele in the cell or tissue type that determines the early lethality of null mutants. However, we observed that animals that were heterozygous for the Pon3 null allele exhibited a phenotype; namely, the body weight and placental weight were ~5–10% lower than wild-type littermates at E17.5, and we considered that this may be due to imprinting. Hence, we studied body and placental weight in the mating of heterozygous mothers and wild-type fathers and wild-type mothers and heterozygous fathers. Body weight and placental weight were similar among the heterozygotes irrespective of the parent of origin of the mutant allele, which is not supportive of imprinting.

Previous evidence that PON3 acts as an antioxidant was somewhat indirect. It had been shown that PON3, but not PON2, protected low-density lipoprotein from copper-induced oxidation (4). Moreover, a mouse model of oxidative stress had suggested an antioxidant role for PON3. Mice given carbon tetrachloride demonstrate hepatotoxicity due to the conversion...
of carbon tetrachloride to chloride radicals in the liver (11). Mice expressing circulating recombinant human PON3 demonstrated evidence of reduced liver damage and reduced depletion of other hepatic antioxidant enzymes compared with controls. However, these previous findings used model systems and did not directly address the effect of oxygen. Using A549 cells, which are proposed as a model for type II pneumocytes (14), we found that knocking down endogenous PON3 had no effect on total antioxidant capacity in the presence of 2% oxygen. However, in the presence of 21% oxygen, PON3 knockdown was associated with reduced total antioxidant capacity. The fact that this was observed only in the presence of elevated oxygen tension suggests that the presence of endogenous PON3 prevented the consumption of other antioxidants (such as reduced glutathione), as was observed in the mouse liver exposed to carbon tetrachloride (11). This is the first direct evidence, to our knowledge, that PON3 protects against the effect of increasing partial pressure of oxygen in human cells. Moreover, stable knockdown of PON3 reduced proliferation in the same cell line in ambient oxygen levels.

In conclusion, we had observed that upregulation of PON3 in late fetal life was conserved between the rat, sheep, and human. We hypothesized that this was an important preparatory process to reduce oxidative stress following the exposure of the infant to the elevated oxygen levels of extrauterine life. We have now found that PON3−/− mice exhibited increased early embryonic and neonatal mortality, and PON3 knockdown in a human cell line reduced cell proliferation and total antioxidant capacity in ambient oxygen levels. These data add further support to the proposal that low levels of PON3 may contribute to morbidity and mortality in preterm human infants and that administration of synthetic PON3 may have potential as a means of reducing oxidative stress in neonatal intensive care.

GRANTS
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DISCLOSURES
G. Belteki, S. L. Kempster, and D. Licence have nothing to declare. D. S. Charnock-Jones and G. C. S. Smith are named inventors on a patent application filed by Cambridge Enterprise relating to the use of paraoxonase 3 as a therapeutic product.

AUTHOR CONTRIBUTIONS
S. L. Kempster, D. S. Charnock-Jones, and G. C. S. Smith are named inventors on a patent application filed by Cambridge Enterprise relating to the use of paraoxonase 3 as a therapeutic product.

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