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Xenon induces transcription of ADNP in neonatal rat brain☆

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ABSTRACT

Xenon and other inhalational agents induce cell and organ protection through different and only partially elucidated molecular mechanisms. Anesthesia induced or pharmacologic preconditioning is a recognized mechanism of cell protection. In this study we explored the gene transcription of activity-dependent neuroprotective protein (ADNP) in neonatal rat brain as consequence to xenon exposure, comparing the noble gas to nitrogen. Seven-day-old Sprague Dawley rats were exposed for 120 min to 75% xenon and 25% oxygen or control condition consisting of 75% nitrogen and 25% oxygen (Air). ADNP was found to be differentially expressed by SSH, validated by Relative Real-Time PCR (RT-PCR) and confirmed by western blot and immunohistochemistry. The differential expression of ADNP in the rat neonatal brain may account for the preconditioning and neuroprotective effects exerted by gas xenon.

Interest in xenon has evolved from the recent description of its neuroprotective properties to anesthesia induced preconditioning effects in several models of acute neurological injury involving three species (mice, rats, pigs) [12,15,17]. Indeed previous studies have shown that the size of cerebral infarction following temporary cerebrovascular occlusion is reduced when animals are exposed to xenon compared to animals breathing air or other anesthetic gases such as nitrous oxide [1,4,8,12]. The neuroprotective effect of xenon has been reproduced both in vitro and in vivo at sub-anesthetic doses, suggesting that xenon may be used in the treatment of conditions such as stroke or neonatal asphyxia [1,13]. The neuroprotection provided by xenon is long-lasting [10] and it also mitigates anesthesia induced neuroapoptosis during synaptogenesis, the delicate period of brain synapsis growth spurt [2,11].

Inhalational anesthetics seem to induce preconditioning through new protein synthesis and recent studies have shown protective effects in model of neonatal hypoxia ischemia exerted by the glial derived protein ADNP or activity-dependent neuroprotective protein, a key brain protein involved in several cell survival mechanisms, and its active synthetic form NAP, an eight amino acids peptide [5,7]. To gain further insight into the molecular mechanisms underlying the preconditioning effects of xenon we decided to investigate the effects of xenon on ADNP gene transcription and translation.

This study was approved by the Home Office (UK) and conforms to the United Kingdom Animals (Scientific Procedures) Act of 1986. All efforts were made to minimize animal suffering and the number of animals used. Postnatal day seven (P7) Sprague/Dawley rats from two different litters were used. Two cohorts (the experimental and the validation set) of 4 rats each were paired randomly. The control group was exposed to air (75% nitrogen and 25% oxygen) and the treatment group was exposed to xenon (75% xenon and 25% oxygen). Xenon concentration was measured continuously by an in-line gas analyzer (Air Products Model No. 439Xe), while other gases were monitored through infrared gas analyzer (Datex). The temperature of the exposure chambers was maintained at 37 degrees C in a water-bath. After two hours of exposure rats were immediately decapitated, their brains isolated in ribonuclease-free conditions, snap frozen in liquid nitrogen and stored at −80 °C.

Total RNA was isolated from the experimental rat brains according to a method described by Chirgwin et al. [3]. Tissue samples

☆ Inhalational anesthetics and noble gases (xenon, helium) have shown to induce protective genes involved in anesthesia preconditioning (APC) in the brain, heart and other organs. The aim of this study was to evaluate the induction by gas xenon of speculative protective gene ADNP at the transcriptional level, proteomic and immunohistochemistry level.

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were homogenized in an Ultra–Turrax homogenizer in the presence of a denaturing guanidium thiocyanate buffer.

The cDNA suppressed library was constructed as the method described originally by Diatchencko et al. [6] and then by Traina et al. [20].

Poly(A)+ RNAs isolated using the PolyA-tract® mRNA Isolation System (Promega) were reverse-transcribed using ImProm-II™ Reverse Transcription System (Promega). The procedure requires 0.5–1 μg of poly (A)+ RNA from both mRNA populations that are first converted in cDNAs prepared from the treated and control samples. The cDNAs were used as tester and driver, respectively, for the forward subtraction and vice versa for reverse subtraction. Tester and driver cDNAs were hybridized, and the hybrid sequences were removed. Consequently, the unhybridized cDNAs represent fragments corresponding to modulated or on/off-switched sequences. The efficiency of subtraction was estimated by hybridization and PCR analyses.

Hybridization analyses were carried out with Southern blot of cDNAs derived from subtracted and unsubtracted libraries. cDNA was used as probe, derived from forward- and reverse-subtracted libraries after labeling with DIG DNA Labelling Kit (Roche, Mannheim, Germany).

To eliminate the background due to the presence of the adapter sequences (BD PCR-Select cDNA Subtraction Kit; BD Biosciences) in the probes, a high concentration (3 μg/ml) of oligonucleotides corresponding to the nested primers and the complementary sequences (competitors) previously used for the libraries construction has been added into the pre-hybridisation and hybridisation solution. Moreover, the adaptors have been removed by digesting cDNAs with RsaI restriction enzyme prior to probe labelling.

PCR analysis consists in the amplification of ribosomal and the housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA in order to evaluate its expression in subtracted and unsubtracted cDNAs. Aliquots of the samples were taken after 30, 31, 32, and 33 cycles of PCR amplification and the products were analyzed by agarose gel.

Amplified cDNA sequences from the forward and reverse subtraction were directly inserted into a T/A-cloning vector using the TOPO TA Cloning Kit (Invitrogen, Milano, Italy). Positive clone analysis was carried out by differential screening: two identical blots were created and then hybridized with digoxigenin-labeled forward and reverse libraries probes. In order to quantify the difference in signal intensity, spots were scanned using an image scanner (Amersham BioScience). Signals were then quantified with the Image Master 2D platinum software Version 5.0 (Amersham BioScience). Clones showing an on/off hybridization signal were sequenced by the dideoxy chain terminator method with an automated DNA sequencer (MGM, Institute of Molecular Genetics and Medicine, Pisa, Italy).

Database searches were performed using the National Center of Biotechnology Information server (NCBI; http://www.ncbi.nlm.nih.gov) with BLAST algorithms at the European Bioinformatics Institute server (EBI; http://www.ebi.ac.uk) and at the Rat Genome Database (RGD; http://rgd.mcw.edu). SSH raw data validation was performed by semi-quantitative RT-PCR on total RNA used for library construction.

PCR products were separated on 2% agarose gel electrophoresis and band intensities were analyzed by the Quantity One® software version 4.4.1. (Bio-Rad). Validation of selected sequences by relative Real-Time PCR (MGM, Institute of Molecular Genetics and Medicine, Pisa, Italy) was performed using 7900HT FAST Real-Time PCR Sybr Green I Dye-method (Applied Biosystems) according to the manufacturer's instructions.

The results were corrected and efficiency controlled by equal loading of total RNA evidenced after ethidium bromide staining and by hybridization with GAPDH housekeeping gene. 4 replicates were analyzed. A delta-delta CT method for calculation of fold-differences was used [9].

To analyze the difference in protein translation we performed a western blot quantitative analysis on activity-dependent neuroprotective protein (ADNP). The pups were randomly chosen from those left from the SSH gas exposure. Two pups per each time point were exposed to xenon or air. The pups were humane-killed under thiopental anesthesia at 0, 2, 8 or 24 h after xenon treatment and the all cortex harvested for western blot as fully described previously [13].. The mice monoclonal antibody directed against ADNP (1:1000 dilution; BD Bioscience, Erembodegem, Belgium) and α-tubulin (1:2000 dilution; Sigma, Poole, UK) were used as primary antibodies. Horseradish peroxidase (HRP)-conjugated IgG (1:2000, New England Biolab) was used to detect the primary antibodies. The bands were visualized with the enhanced chemiluminescence (ECL, Amersham Biosciences, Little Chalfont, UK) system and differences in signal emission analyzed (Quantity One® software version 4.4.1).

Immunohistochemistry was performed to evaluate and confirmed the relationships between gene transcription, protein translation and localization at cellular/nuclear level.

Another four P7 rats from those left from the SSH exposure to xenon ($n = 2$) or air ($n = 2$) were sacrificed 24 h after exposure. Rats were euthanized with thiopental and then perfused with 4% paraformaldehyde. The brains were removed, post-fixed overnight in perfusate, and processed for paraffin embedding. Serial coronal sections (6-μm thick) were cut with a microtome, collected on super-frost slides and dried overnight at 37 °C. Paraffin sections were stained with antibody against activity-dependent neuroprotective protein (ADNP) as per manufacturer indications (1:250 dilution; NB110-40556, Novus Biologicals, Littleton, CO). Visualization of primary antibody was done with VECTASTAIN® Elite ABC Kits (Vector Laboratories, Burlingame, CA; USA), and diaminobenzidine tetrahydrochloride (DAB) as substrate (K3466, DakoCytomation; Denmark), which is based on an indirect streptavidin–biotin method. Slides were counterstained with hematoxylin. Staining intensity and morphologic qualitative assessment was carried out by two blinded independent observers using a 40× magnification in two separate evaluation areas for each treated animal.

Real-Time PCR data were subjected to two models of statistical analysis: a randomization test performed using REST 2005 version 1.9.12. software (©Corbett Research) [14,21], followed by paired Student *t*-test [9,14,21]. Post hoc analysis was performed with Student–Newmann–Keuls. A P value <0.05, obtained by both statistical analyses, was considered significant.

Western Blot analysis was performed with ANOVA and post hoc Student–Newmann–Keuls. A P value was considered significant if less than 0.05.

PCR-based suppression subtractive hybridization was used to identify differential gene expression of ADNP in response to xenon exposure in neonatal rat brain. The efficiency of subtraction was evaluated by PCR amplification of the housekeeping gene for GAPDH. The transcripts of the housekeeping gene were greatly reduced and detectable as a faint band in the subtracted sample after 33 amplification cycles of PCR, while they were clearly detectable in the unsubtracted sample at only 31 amplification cycles (data not shown). These results were confirmed by Southern blot analysis of cDNAs derived from unsubtracted and subtracted libraries.

49 clones were differentially screened (Table 1) from the forward- and reverse-subtracted libraries as positive clones. The screening of the positive clones was carried out by colony PCR, using the same nested primers as were used for the library amplification. Two identical blots were created by spotting PCR products