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Effects of Isoflurane on *Nfκb p65*, *Gadd45a* and *Jnk1* Expression in the Vital Organs of CBA/CA Mice

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Abstract. *Background: Isoflurane is administered to patients during general anaesthesia to diminish the effects of surgical invasion. Materials and Methods: CBA/CA mice were exposed to 2% isoflurane during general anaesthesia. Gene expressions were measured 6 and 12 hours later by quantitative real-time PCR on total RNA isolated from the lung, liver and kidneys of the animals. Expressions of growth arrest and DNA-damage-inducible 45 alpha (Gadd45 α), Jun N-terminal kinase 1 (Jnk1) and nuclear factor of kappa light polypeptide gene enhancer in B-cells p65 subunit (Nfκb p65) were analyzed. Results: Gadd45α and Jnk1 showed significant inverse expression changes in the different tissues compared to the Nfκb p65. The length of anaesthesia also modified the gene induction. Conclusion: Isoflurane has significant modulatory effect on the transcription of genes regulating inflammation and apoptotic signalling.*

Isoflurane, 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, is one of the most commonly used volatile anaesthetics in clinical practice for general anaesthesia. It is considered to be a less hepatotoxic volatile anaesthetic than halothane since it undergoes quantitatively less metabolism to form toxic reactive intermediates (1-2).

Postoperative pulmonary and renal excretion of isoflurane and its metabolites have been studied extensively (3-4). During isoflurane administration, alveolar concentration and uptake were accurately measured in patients undergoing surgery (5-6). Of the isoflurane taken up, 95% was recovered after surgery in exhaled air. Postoperative increase in urinary excretion of inorganic fluoride was less than 0.2% of the fluoride taken up as isoflurane. (6). In patients with 3 minimal alveolar concentration/hour (MAC/h) of exposure to isoflurane, peak serum fluoride levels were approximately 3.6-5.08 μM (7-8). Approximately 50 μM is the fluoride level necessary to produce early evidence of renal toxicity (7). Minimal biotransformation and the low blood/gas partition coefficient of isoflurane results in minute amounts of isoflurane storage in lipid tissue after anaesthesia with subsequent metabolism of intermediary or toxic metabolites (1).

In recent years some research has led the way towards molecular genomic evaluation of volatile anaesthetics, including isoflurane. *In vitro* and *in vivo* investigations revealed the effects of isoflurane exposure on expression of genes for inflammatory cytokines of alveolar epithelial type II cells, alveolar macrophages, neutrophils and mononuclear cells (9-13). These studies mainly focused on the analysis of the transcription regulation of early responding and inflammatory genes. Hamaya *et al*. concluded that isoflurane anaesthesia induced significant expression of *C-jun* and *C-fos* in heart, liver, kidney and brain of Sprague-Dawley rats in dose- and time-dependent manners (13). Other recent results show that similarly to sevoflurane, isoflurane has a potential down-regulating effect on interleukin 8 (IL 8) and nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB) expressions of human monocytic THP-1 cells induced by tumor necrosis factor alpha (TNFα) (14). The NFκB intracellular signalling pathway plays an important role in the regulation of acute and chronic inflammation and carcinogenesis (15). NFκB p65 is a subunit of the NFκB heterodimer that is the central transcription factor for a wide range of genes participating in the regulation of apoptosis and cell survival. It is related to single-strand DNA damage by growth arrest and DNA-damage-inducible 45 alpha (*Gadd45α*) gene. GADD45α

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production is strongly linked to the JNK cascade of which Jun *N*-terminal kinase 1 (*Jnk1*) is an important member (16-17). In the present study, we evaluated the expressions of mouse genes *Gadd45α, Jnk1* and *Nfκb p65* as a function of time of isoflurane anaesthesia with the aim of clarifying the kinetics of DNA damage-inducible mRNA transcription influenced by anaesthetic exposure.

Materials and Methods

Conventionally kept five-week-old CBA/Ca (H-2^k) inbred mice (sensitive to chemical carcinogen exposure) weighing between 20-23.5 g were used. Four groups were maintained containing twelve animals in each. The first two groups were exposed to isoflurane: the first group for 1 hour and the second for 2 hours in a plastic chamber with a volume of approximately 3 l, connected to an anaesthetic machine with a half opened system that is generally used for anaesthesia of small mammals. Anaesthesia was administered with a forane vaporizer calibrated for isoflurane at concentration of 2% (1.5 minimum alveolar concentrations) for 1 hour, employing 90% oxygen at 3 l/min as the carrier gas. The third and fourth groups were control groups. For the controls, 90% oxygen at inhalation of 3 l/min was maintained for 1 hour or 2 hours.

Six animals from each groups were autopsied 6 hours after anaesthesia and six mice 12 hours after anaesthesia. All the animals in the differently exposed groups were treated humanely.

Lung, liver and kidneys of the animals were removed during autopsy. Tissue samples of each organ were pooled according to groups. Total RNA was isolated from the organs with MagNA Pure Compact automatic nucleic acid isolation system (Roche, Berlin, Germany) using MagNA Pure Compact isolation kits (Roche) according to the manufacturer's instructions. The quality of the isolated RNA was checked by absorption photometry at 260/280 nm. The optical density of the RNA was between 1.9 and 2.1.

High purity total RNA was used in quantitative real-time PCR using a LightCycler 2.0 instrument (Roche, Berlin, Germany). Reverse transcription and nucleic acid amplification was carried out with one-step LightCycler RNA Amplification kit (Roche) using CYBR green fluorescent labelling. Primers for *Nfκb p65, Gadd45α, Jnk1* and *Hprt* were selected from a primer finder database (www.applied-science.roche.com) and were synthesized by TIB Molbiol, ADR Logistics, (Roche Warehouse, Budapest, Hungary) and were the following: *Nfκb p65* forward: 5'CACTGCTCA GGTCCACTGTC3', reverse: 5'CTGTCACTATCCCGGAGTTCA3'; *Gadd45α* forward: 5'CTGCCTCCTGGTCACGAA3', reverse: 5'TTGCCTCTGCTCTCT TCACA3'; *Jnk1* forward: 5'AACTGT TCCCCGATGTGCT3', reverse: 5'TCTCTTGCCTGACTGGCTTT3'; *Hprt* forward: 5'TCCTCCTCA GACCGCTTTT3', reverse: 5'CCT GGTTCATCATCGCTAATC3'.

All RNA samples were run in triplicates in 20 μl optical capillaries. The PCR reaction mix contained: 1 μl of the tissue RNA sample, 2 μl of the primer mix of the forward and the reverse primers at 0.5 μM final concentration, 8 μl of PCR grade H_2O, 0.4 μl of the RT-PCR Enzyme Mix, 3 μl of the Resolution solution, 1.6 μl of the MgCl2 stock solution and 4 μl of the LightCycler RT-PCR Reaction Mix SYBR Green I.

Thermal cycling conditions for the PCR were the following: 1 cycle for reverse transcription at 55˚C for 10 min, denaturation at 95˚C for 30 s followed by amplification of 45 cycles of three steps: denaturation at 95˚C for 0.01 s, annealing at 55˚C for 15 s and extension at 72˚C for 4 s. Melting curves were gained from 1 cycle of 95˚C for 0.01 s, 55˚C for 30 s and 95˚C for 0.01 s as melting with continuous detection. Fluorometric detection was carried out using a 530 nm channel according to SYBR green fluorescent labelling. For normalization, the average intensity of the three technical replicates of each PCR reaction was taken. The absolute mRNA content for Gadd45α, *Nfκb p65*, *Jnk1* and *Hprt* of the tissues was determined and gene expression alterations were calculated relative to *Hprt*. Statistical analysis was carried out with STATA IC 11 for Windows (StataCorp LP, Texas, USA). Data are expressed as the mean±SD and were analysed by two-sample Student's *t*-test. Differences were considered significant at $p \leq 0.05$.

Results

Significant expression changes were found in the lung and the kidney and were dependent both on the duration of isoflurane anaesthesia and the time subsequent to the exposure (Figure 1). Significant expression changes were only found in the lung and kidney tissues, and none of them reached the level for significance in the liver. Lung tissues showed significant changes with 1 hour and 2 hours of isoflurane exposure depending on the gene in question. *Nfκb p65* was overexpressed only after 2 hours exposure but this marked up-regulation was seen at 6 and 12 hours after anaesthesia. Conversely down-regulation of *Gadd45α* and *Jnk1* was seen in the groups exposed for 1 hour. The trend of this under-expression was also seen after 2-hour exposure at 6 and 12 hours after anaesthesia in the case of the *Jnk1* gene, but was significant only at the 6-hour time point.

The liver tissues showed only one considerably significant expression reduction which was for the *Gadd45α* gene after 1 hour of anaesthesia and at the 12-hour time point.

In the kidneys, interestingly, the expressions of *Jnk1*, *Gadd45α* and the *Nfκb p65* were found to show just the opposite correlations that were seen in the lung. *Nfκb p65*was found to be down-regulated in the groups receiving the longer exposure, at both time points, while *Gadd45α* and *Jnk1* were markedly overexpressed in the 1-hour exposure groups: after 6 and 12 hours in the case of *Gadd45α* and after 6 hours in the case of *Jnk1*.

Discussion

NFκB is a sequence-specific transcription factor and is one of the key regulators of inflammatory and cell survival mechanisms. NFκB consists of a number of closely related protein dimers that bind a common sequence motif known as the κB site. Various homo- and heterodimers from p65 (RelA) and the c-Rel or p50 protein subunits form NFκB which acts on the canonical activation pathway dependent on IκB kinase activation. The pathway is triggered by inflammatory cytokines *via* lipopolysacharide receptors and the toll-like receptor signals induced by microbial, viral and chemical exposure (15, 18). The second, alternate, pathway affects NFκB2/p100 dimerized preferentially from RelB subunits and triggered by

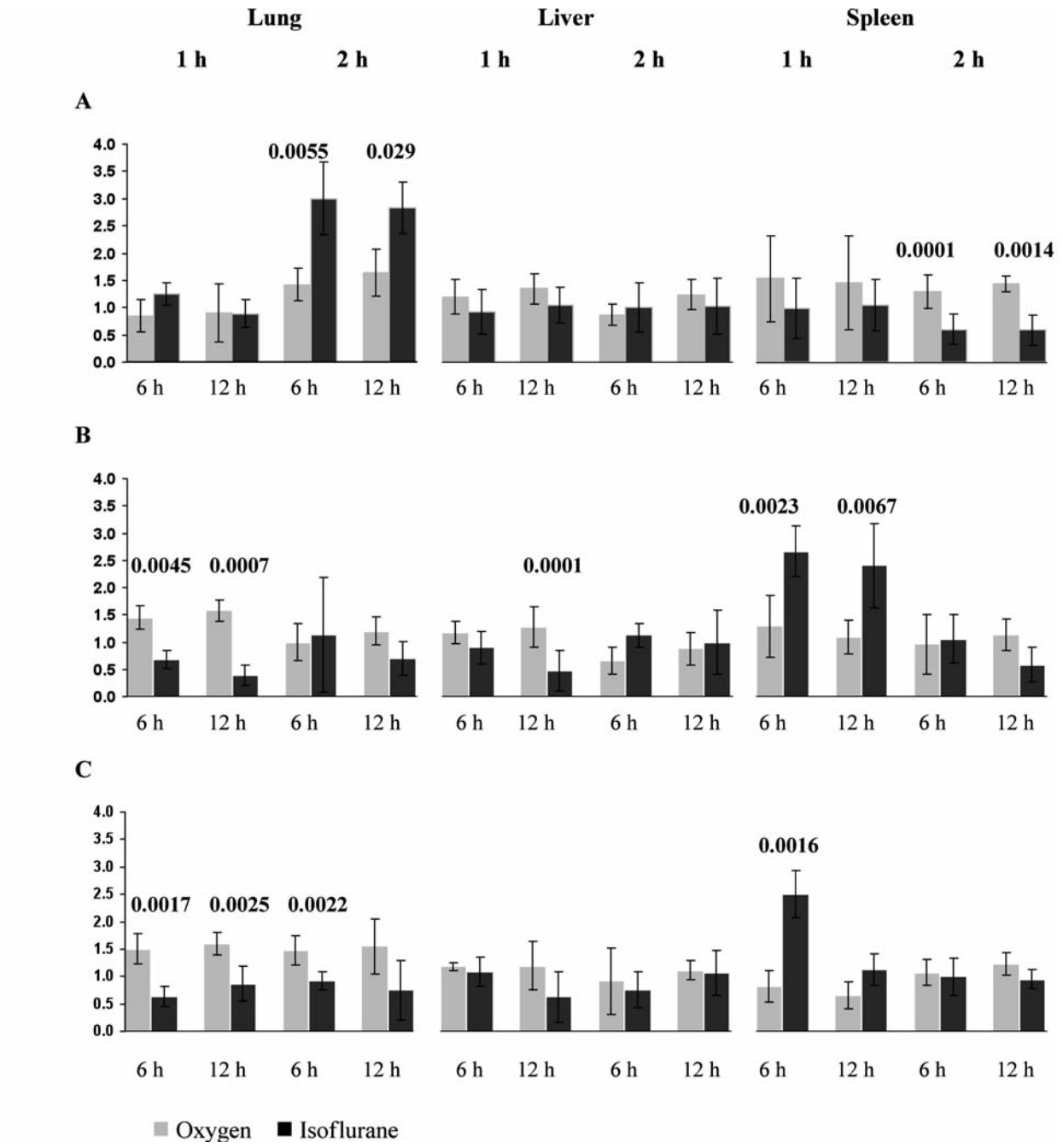

Figure 1. *Gene expression of Nfκb p65 (A), Gadd45α (B) and Jnk1 (C) relative to Hprt in the lung, liver and kidney tissues 6 and 12 hours after 1 or 2 hours of isoflurane anaesthesia. Data represent the mean value of expressions from the replicates with the confidence intervals and the significant p-values.*

the TNF cytokine family (19). Each NFκB dimer has distinct regulatory functions, however, their target genes are common, positively regulating the cell cycle, immunoregulatory and anti-apoptotic genes. The NFκB signalling and the Jnk1 survival pathways are connected *via* GADD45α and mitogen-activated protein kinase 4 (MEKK4). *Gadd45α* transcript levels increase following stressful growth-arrest conditions and DNA-damaging agents. GADD family members interact with MEKK4 that is the upstream kinase of Jnk1, facilitating the escape from programmed cell death (17, 20).

Our results demonstrate that the effects of isoflurane anaesthesia on the expression of a gene network participating in inflammation and cell death mechanisms show large differences in the different parechymal organs such as lung, liver and kidneys of CBA/CA mice. The expression was generally dependent on the length of the anaesthetic exposure and on the time since anaesthesia; however, the greatest differences were seen according to the type of tissue. Interestingly, lung and kidney tissues responded inversely to isoflurane: in the case of *Nfκb p65*, overexpression in the lungs and, at the same time, underexpression in the kidneys. In the case of the *Gadd45α* and *Jnk1*, the kidneys showed overexpression while the lung tissues exhibited down-regulation of these genes. This reverse correlation could be explained by the fact that while the lung is dominantly exposed to isoflurane molecules, with minimal metabolism in the lung tissues due to low blood/gas solubility and quick clearance, the small but detectable inorganic ionic and non-ionic isoflurane metabolites reach highest concentrations in the kidney tissues because of their urinal excretion and locally can cause oxidative damage of DNA (8, 21, 22). The differing inducibility and kinetics of the investigated genes can be also seen as a longer exposure resulted in significant and sustained dysregulation of *Nfκb p65*, while *Gadd45α* and, in particular, *Jnk1* activation was seen after 1-hour anaesthesia and evoked a response but then the dysregulation was silenced faster. Based upon these findings, isoflurane seems to have a time-dependent and significant effect on the transcription modulation of genes regulating inflammation and apoptotic signalling.

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