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OGG1 mRNA expression and incision activity in rats are higher in foetal tissue than in adult liver tissue while 8-oxo-2′-deoxyguanosine levels are unchanged

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Abstract

This study was set up to investigate the relationships between the formation and removal of DNA damage in form of 8-oxodeoxyguanosine (8-oxodG) in neonatal (day 16 of gestation) as compared to adult rats. The hypothesis addressed was whether the rapidly dividing foetal tissue has an enhanced requirement of DNA repair providing protection against potentially mutagenic DNA damages such as 8-oxodG. The activity of the primary 8-oxodG-repair protein OGG1 was measured by a DNA incision assay and the expression of OGG1 mRNA was measured by Real-Time PCR normalised to 18S rRNA. The tissue level of 8-oxodG was measured by HPLC-ECD. We found a 2–3-fold increased incision activity in the foetal control tissue, together with a 3–15-fold increase in mRNA of OGG1 as compared to liver tissue from adult rats. The levels of 8-oxodG in the foetal tissue were unaltered as compared to the adult groups. To increase the levels of 8-oxodG, the rats received an injection (i.p.) of the hepatotoxin 2-nitropropane. The compound induced significant levels of 8-oxodG in male rat livers 5 h after the injection and in the foetuses 24 h after the injection, while the female rats showed no increase in 8-oxodG. The incision activity was slightly depressed in both male and female liver tissue and in the foetal tissue 5 h after the injection, but significantly increased from 5 to 24 h after the injection. However, it did not reach levels significantly above the control levels.

In conclusion, this study confirms that foetal tissue has increased levels of OGG1 mRNA and correspondingly an enhanced incision activity on an 8-oxodG substrate in a crude tissue extract.

Keywords: Rat OGG1 expression; DNA repair activity; 8-OxodG; Proliferation; 2-Nitropropane

1. Introduction

Living cells require a correct function of thousands of proteins, which each can be modified by a mutation at many different sites of its gene. Thus the DNA sequences must be passed on undamaged if cell progeny is to have a fair chance of survival. It is believed that an organism cannot survive the natural rate of damage to its DNA without enzymatic mechanisms to repair damaged sites [1].

The free radical theory of ageing, that was proposed in 1956 [2], suggests an age-dependent accumulation of DNA modifications connected with the symptoms of ageing and since then several studies have addressed the relationships between DNA damage, repair and ageing and many have supported the theory (reviewed

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in [3]). Increased levels of mutagenic adducts might also be of concern in rapidly proliferating foetal tissue, especially because DNA replication enhances the risk of fixation of promutagenic DNA lesions and thus may contribute to critical mutations.

DNA repair during normal embryogenesis is clearly important since many of the phenotypes of heritable DNA repair deficiency syndromes include evidence of significant developmental disruption such as the neurological abnormalities manifested in xeroderma pigmentosum, ataxia telangiectasia and Cockayne syndrome. UV irradiation has been shown to increase nucleotide excision repair by 2–3-fold in primary embryonic mouse skin fibroblasts cultures, and by 10-fold in the extra embryonic mesoderm as compared to adult mouse skin fibroblasts, measured by unscheduled DNA synthesis [4]. The AP endonuclease that is responsible for base excision repair of abasic sites, was expressed in foetal rat tissue in eight somatic sites examined during foetal development, with very high levels in the thymus, liver and developing brain [5]. Levels of thymine glycol glycosylase have also been reported increased in rat embryonic brain compared to adult brain tissue [6]. So it seems reasonable to hypothesise that the embryonic tissue displays a higher degree of DNA repair in general. This was tested for the base excision repair of oxidised guanine (8-oxodeoxyguanosine; 8-oxodG) in the present work.

The interest in the DNA oxidation product 8-oxodG is the result not only of its suitability as an oxidative stress marker but also of its mutagenicity and carcinogenic potential and its proposed involvement in ageing [3,7–9]. The level of a given modification in DNA isolated from tissue or cells reflects the balance between the induction rate of the modification and the DNA repair. It is thus a marker of the steady state of oxidative DNA modifications. The main DNA repair pathway for the 8-oxodG lesion is base excision repair by the 8-oxodG glycosylase OGG1 [10], which is a bifunctional glycosylase that excises the damaged base and subsequently incises the DNA strand at the site of the lesion. It has been proposed that measurements of the OGG1 incision capacity would be another useable biological marker of cellular oxidative stress when chemical carcinogens or other environmental agents in the target tissues have induced damage.

Xenobiotics of diverse structures but also metabolites formed in the maternal tissue have been shown to readily cross the placenta in mice [11] and transplacental exposure has proved to induce tumours in the offspring in at least eight animal species [12] often with a high incidence and short latency. Examples of foetal exposures and effects include high levels of PAH-induced DNA adducts observed in foetal rodents organs [11,13], genital cancers after intrauterine exposure to diethylstilbestrol [14,15], and the thalidomide cases, that also proves that the placenta does not protect foetuses from toxic injuries. The levels of oxidative DNA damage in foetuses have not yet been very well described. However, a growing number of chemicals have been shown to initiate potentially embryopathic oxidative stress [16] and thus oxidation of DNA, proteins and/or lipids. Compounds producing oxidative damage in foetal DNA include phenytoin, thalidomide, some anti-cancer drugs, benzo[a]pyrene, cocaine (summarised in [16]), and the tobacco-specific nitrosamine NNK [17].

The hepatocarcinogen 2-nitropropane (2-NP) has been shown to induce foetal toxicity after repeated intraperitoneal injections (170 mg/kg) to pregnant rats [18]. In rat liver tissue, 2-NP induce oxidative DNA damage possibly through intracellular generation of reactive forms of oxygen and/or 2-NP radicals by cytosolic sulfotransferases [19–22], but whether the same mechanism is responsible for the foetal toxicity is not known.

In this study, the levels of 8-oxodG and OGG1 in terms of mRNA expression and incision activity in foetal and adult rat tissue were compared, both under normal conditions and after induction of oxidative damage by means of 2-NP administration.

2. Materials and methods

2.1. Reagents

Reagents were purchased from Sigma unless otherwise stated.

2.2. Animals and treatment

The study included three groups of nine Wistar rats each; two groups of adult rats (adult male and adult female), and one group of adult pregnant rats on day 16 of gestation. The animals received standard laboratory

diet (Altromin 1314) and tap water ad libitum. They were housed three to a cage with aspen wood bedding (Finntapwei, Finland) in an environmental controlled animal facility operating on a 12 h dark/light cycle and 55% humidity.

In order to study the effect of 2-nitropropane (2-NP), the rats in each group were randomly allocated in a control group and two treatment groups each of three rats. The treatment groups were injected intraperitoneal (i.p.) with 100 mg/kg body weight 2-NP (Aldrich) in 5 ml/kg corn oil. The two groups of treated rats were killed 5 h or 24 h after the injection respectively. Control rats received vehicle only (i.p. injection of 5 ml/kg corn oil). They were killed 5 h after the injection. The livers and in case of the pregnant rats the foetuses were dissected and immediately frozen in liquid nitrogen. The tissue was stored at −80 °C until use.

Tissue extracts and RNA/DNA extractions were made from liver tissue from the adult and pregnant rats whereas the entire foetuses were used due to the small size of the foetuses and the amount of tissue needed.

2.3. Measurement of incision activity

The rOGG1 incision activity was measured in crude tissue extracts using a ^{32}P-labeled synthetic oligonucleotide (DNA Technology, Aarhus, Denmark) containing a single 8-oxodG lesion as described in [23], except for the amount of extract that was increased to a volume corresponding to 30 μg total protein. Extracts were incubated with ^{32}P-labelled substrate in a total volume of 20 μl. The cleavage products were separated by 20% denaturing PAGE, followed by phosphor imaging of the dried gel using a Fujix Bio-Imaging Analyser System BAS2000^{TM}.

Upon incubation of the oligonucleotide substrate with crude rat tissue extracts, we detected the formation of a 14′ mer fragment (13′ mer with an unsaturated abasic residue at the 3′ end), consistent with the β-elimination mechanism of OGG1. The size of the fragment was verified on the gel by comparison with an oligonucleotide treated with the *E.coli* repair enzyme Fpg (Pharmingen International, San Diego, CA). The formation of the incision product was dependent upon the total protein concentration for the extracts indicating that the reaction had not reached saturating conditions (not shown). The incision activity was calculated as the amount of radioactivity in the band corresponding to the damage specific cleavage product over the sum of radioactivity of the bands corresponding to the intact and the cleaved oligonucleotide. The incision activity of each sample was then normalised to the average ratio of three control extracts loaded on every gel.

2.4. Measurement of rOGG1 mRNA expression

The rat OGG1 mRNA was quantified by Real-Time PCR using the LightCycler System (Roche Molecular Biochemicals, Mannheim, Germany), by normalisation to the endogenous reference 18S rRNA as described previously [24]. Briefly, total RNA was extracted from the liver or foetal tissue and reversed transcribed. For the LightCycler reactions, the cDNA preparation was mixed with water, $MgCl_2$ and LightCycler master mix (FastStart DNA Hybridisation Probes, Roche Molecular Biochemicals, Mannheim, Germany). Half of this reaction mixture was mixed with the rOGG1 primers and probe while the other half was mixed with the rRNA probe and primer solution. The samples were amplified by 45 heating and cooling cycles with a single fluorescence measurement in each cycle. The individual level of initial target cDNA was expressed as the difference in C_T (i.e. the calculated fractional cycle number at which the PCR product crosses the threshold of detection) between rOGG1 and 18S in the two parallel samples (ΔC_T). The relative amount of rOGG1 mRNA normalised to 18S rRNA was given as $2^{-\Delta C_T}$. The average of three duplicates on two different days was used.

Rat OGG1 primers were selected to span the intron region between exons 2 and 3 in the *rOGG1* gene. Control experiments without reverse transcriptase enzyme in the reaction mixture showed no PCR products, thus verifying that products in the normal reactions originated from the RNA only.

2.5. 8-oxodg analyses

The 8-oxodG analyses were performed as described [24]. In short, the DNA from approximately 100 mg liver or foetal tissue was extracted and precipitated as described by Nakae et al. [25] and Asami and Kasai [26]. The DNA was resuspended in 10 mM

Tris/0.1 mM desferrioxamine prior to enzymatic hydrolysis with nuclease P_1 and alkaline phosphatase (Boehringer Mannheim, Germany). The deoxyribonucleotides were then treated with DOWEX 1× 8-400 ion-exchange resin (The Dow Chemical Company, Midland, MI) and finally filtered through a Micropure-EZ filter (Millipore, Bedford, MA). The levels of 8-oxodG and dGuo were measured using an HPLC system with electrochemical and UV detection. The samples were extracted in duplicate and each was injected twice. Peak areas were used for calculations. Calibrations curves were run together with each batch of samples.

2.6. Statistics

The groups were compared by means of analysis of variance (ANOVA), followed if significant by a least significant difference test (LSD-test) for identification of difference between the groups. Probability values (P-values) <0.05 were considered statistically significant.

3. Results

3.1. Incision activity

Fig. 1 shows the results of rOGG1 incision activity in the four groups with three treatment groups each. The activity was significantly increased by two–threefold in the foetal tissue as compared to the activity in the liver tissue from the corresponding male, female and pregnant groups.

The 2-NP treatment did not significantly increase the incision activity in any of the groups relative to the controls (Fig. 1). We saw a small but insignificant decrease in incision activity in three groups (adult male, adult female and foetus) 5 h after the 2-NP treatment. The incision activity in these groups

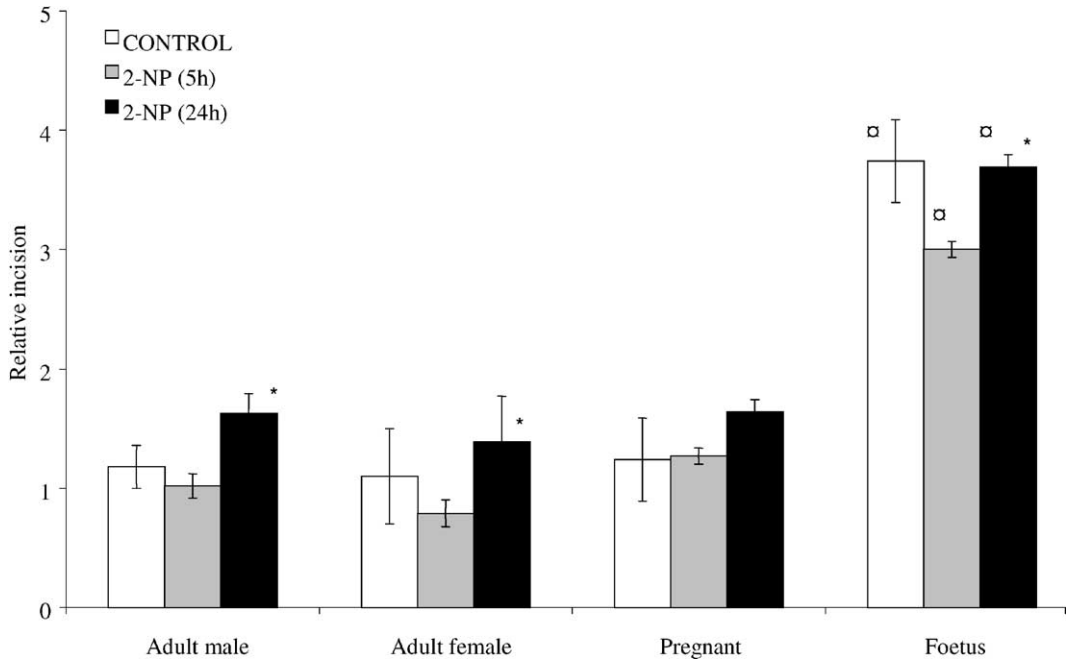

Fig. 1. Quantification of the 8-oxodG-incision activity in crude tissue extracts from adult male, female, and pregnant rat livers, and rat foetuses. Each group consists of a control (white columns) and two 2-NP treatment groups: 5 h (grey columns) and 24 h (black columns) after 2-NP injection. The incision assay and the method for quantification are described in Section 2. Results are presented as the mean with S.D. (bars). $N = 3$ for each group. ¤: Statistically different ($P < 0.05$) from the corresponding treatment in the other groups. ∗: Statistically different from 2-NP (5 h) within the same group.

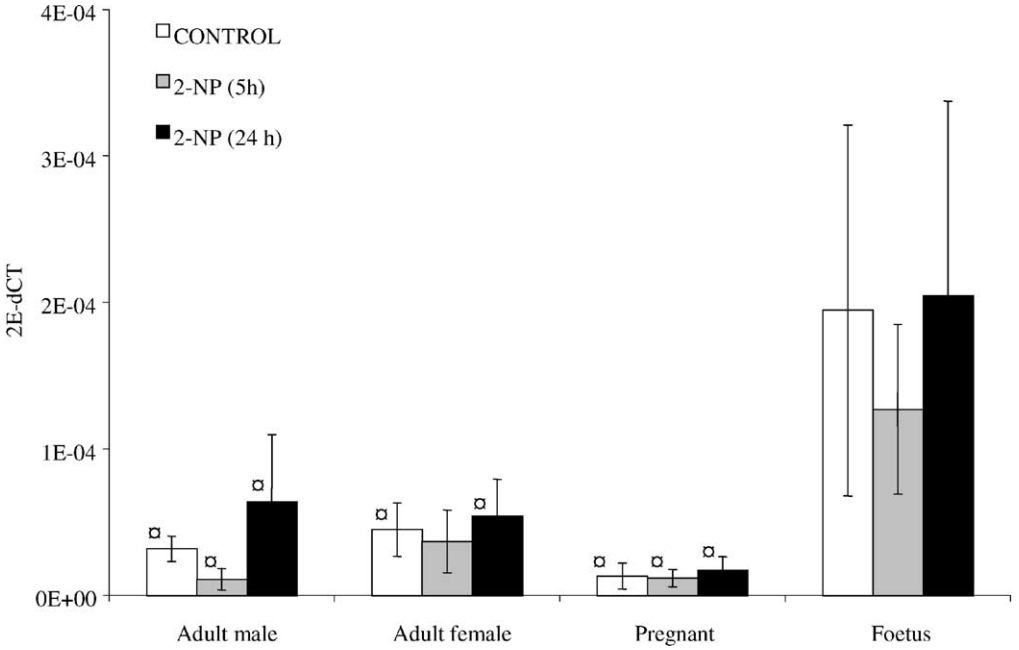

Fig. 2. Expression of OGG1 mRNA relative to 18S rRNA in total mRNA extractions from adult male, female, and pregnant rat livers, and rat foetuses. Each group consists of a control (white columns) and two 2-NP treatment groups: 5 h (grey columns) and 24 h (black columns) after 2-NP injection. The Real-Time PCR analyses are explained in Section 2. Results are presented as mean with S.D. (bars). $N = 3$ for each group. ¤: Statistically different ($P < 0.05$) from the corresponding foetal treatment group.

significantly increased from 5 to 24 h after the treatment ($P < 0.05$). In the pregnant group there was a small increase in incision activity from 5 to 24 h after the 2-NP treatment but it failed to reach statistically significance ($P = 0.11$).

3.2. OGG1 mRNA expression

The levels rOGG1 mRNA expression paralleled the levels of incision activity very exactly. The foetal tissue showed a significantly increased rOGG1 mRNA expression of 3–15-fold relative to the corresponding adult groups (liver tissue only) (Fig. 2), except for the 5 h group which did not reach significance to the adult female group ($P = 0.07$).

Following the 2-NP treatments the expression of the rOGG1 mRNA showed the same biphasic response as the incision activities, although no significant differences were found within the four groups, possibly due to by higher variation.

3.3. Oxidative DNA modifications

The levels of oxidative DNA modifications measured by the ratio of 8-oxodG/dGuo are shown in Table 1. The foetal tissue did not show statistically significant higher levels of 8-oxodG than the liver tissue from the adult groups.

The injection of 2-NP increased the levels of 8-oxodG by 2.3-fold in the liver of male rats after 5 h (Table 1). This level was only slightly decreased from 5 to 24 h after the treatment and still significantly higher than the controls. The foetal tissue showed a statistically insignificance 1.4-fold increase 5 h after the 2-NP injection. At 24 h after the 2-NP dose, the levels of 8-oxodG was further increased to 1.5-fold the control level and reached statistically significance. The adult female groups showed identical levels in the three groups, whereas the levels of 8-oxodG in the pregnant rats was decreased in the treatment groups (0.7- and 0.5-fold, respectively) relative to the control

Table 1
Levels of 8-oxodG/10^6 dGuo in the adult male, female, pregnant, and foetus groups in controls and 5 and 24 h after an intra-peritoneal injection of 2-nitropropane (100 mg/kg)

	8-oxodG/10^6 dGuo		
	Control	2-NP (5 h)	2-NP (24 h)
Adult male (liver tissue)	5.27 (1.32)	12.03 (1.07)*	11.69 (0.82)*
Adult female (liver tissue)	7.01 (2.43)	7.07 (0.42)	6.39 (1.18)
Pregnant (liver tissue)	11.40 (1.57)	7.91 (2.49)	5.13 (1.72)*
Foetus	9.53 (0.67)	13.36 (7.17)	14.26 (3.96)*

Numbers are averages with standard deviations (S.D.) of two measurements on HPLC-ECD.
* Significantly different ($P < 0.05$) from the control in the same group.

group. The decrease was statistically significant only after 24 h.

4. Discussion

The present experiments were conducted in foetal rat tissue at day 16–17 of gestation which is a time characterised by foetal growth and a period in which the foetus is highly susceptible to carcinogenic agents [12]. The study confirmed our hypothesis that the fast dividing foetal tissue had a higher DNA repair activity and that this higher repair activity was able to maintain oxidative lesions in tissue at an unchanged level. The increased DNA repair activity was evidenced both by higher levels of rOGG1 mRNA as well as a higher incision activity. These results are in accordance with the previously mentioned results on other types of DNA repair in foetal tissues [4–6]. There was a good agreement between the mRNA levels and the incision activities in the rat livers and foetuses, suggesting that in the examined situations the incision activity were transcriptional regulated. After induction of oxidative stress by the hepatocarcinogen 2-NP, there was an early reduction of DNA repair followed by up-regulation after 24 h. However, the level of DNA repair did not significantly exceed the control levels.

We found the same basal levels of 8-oxodG in the foetal tissue as in the adult liver tissue. Based on the lower partial pressure of oxygen in the foetuses (PO_2 in the umbilical vein is around 30 mmHg compared to 100 mmHg in arterial blood and 40 mmHg in venous blood [27]) and the high rOGG1 activity found in this study, we had expected to find lower levels of oxidative stress in the foetal tissue than in the liver tissue from adult rats. As the liver tends to display higher levels of 8-oxodG than other organs, noticed both in normal rats [28] and in *ogg1*$^{-/-}$ mice [29], the deviation is not suspected to be due to differences in the tissues (total foetus versus liver tissue from the adult rats). Previous findings in foetal rat tissue have suggested lower basal levels of 8-oxodG as compared to the levels during the late postnatal phase [30] and in mice lower levels of 8-oxodG were found in foetal tissue as compared with maternal organs both at basal levels [31] and after 8-oxodG induction by NNK [17], or AZT [32]. However, a few studies found unaltered levels in foetal rat tissue as compared to postnatal levels [33,34] in accordance with our data.

The present data thus shows that the higher rOGG1 mRNA levels and incision activities in the foetal tissue do not cause lower levels of 8-oxodG than in the adult liver tissue. OGG1 is considered to be the main repair pathway for 8-oxodG and cell free extracts from the ogg1$^{-/-}$ mice have shown no detectable incision activity towards an 8-oxodG containing substrate, suggesting not only that OGG1 is the major 8-oxodG glycosylase in mammalian cells [35], but also that the incision assay used is specific to OGG1 activity. However, similar results were found in an in vitro study of CHO cells stable transfected to over-express the hOGG1 protein, as the steady-state levels of Fpg-sensitive sites were unchanged in all transfected clones despite an up to 10-fold higher enzyme activity on 8-oxodG containing oligonucleotides [36]. This suggests that the kinetics of 8-oxodG in cellular DNA might be more complicated than previously assumed.

OGG1 is not an essential protein as knock out mice lacking the OGG1 protein (*ogg1*$^{-/-}$) have only about two–fold increased levels of 8-oxodG

[35] or of Fpg-sensitive sites [29] in the liver at age 10–15 months. One study though showed up to 7-fold increase in 8-oxodG in liver DNA at 14 weeks of age [37]. The spontaneous mutations rates were only two–three-fold increased in the knock out mice [35,37], and the phenotype was fully viable probably due to a back up function of the nucleotide excision repair system [10]. The explanation why we did not find a simple forward relationship between OGG1 and 8-oxodG is probably hidden in the diverse repair pathways and their mutually mechanisms of regulation which are not revealed.

We used the hepatocarcinogenic compound 2-nitropropane to induce 8-oxodG. 2-NP has been found to be a very potent inducer of oxidative DNA damage in liver tissue (measured in tissue as well as by repair products in the urine), strongly indicating toxicity due to generation of reactive oxygen species [19,38]. Thus 2-NP has been widely used both in vitro and in vivo as a model compound for studies of oxidative DNA damage in liver tissue, however never in foetuses.

The effects of the 2-NP treatment on incision activity showed trends to a biphasic response. A total of 5 h after the injection, the incision activity was slightly depressed in three groups (male, female and foetus), but after 24 h the incision activities were significantly increased by 25–75% relative to the respective 5 h groups (again except the pregnant group ($P = 0.11$)), with a tendency to overshoot the control levels. However, the activities did not significantly exceed the control levels. The same pattern was seen with the rOGG1 mRNA expression, but the changes failed to reach statistically significance. The changes in DNA damage measured by 8-oxodG were very different in the four groups. In the adult male group, the 2-NP induced a 2.3-fold increase within 5 h in accordance with the literature [19,20,39,40], though due to improvements in the HPLC-ECD method the exact numbers were generally lower in this study. The foetal DNA showed an insignificant increase of 40% in 8-oxodG levels 5 h after the 2-NP injection, further increased to 50% after 24 h. Proliferating hepatocytes (i.e. after partial hepatectomy) have shown a higher susceptibility to oxidative DNA damage induced by 2-NP [40] than normal hepatocytes, but the present results suggests that this is not a general trend in rapidly proliferating tissue. The levels of 2-NP-metabolising sulfotransferases might account for the differences [41]. In pregnant mice phenytoin induced 8-oxodG formation in embryonic tissues within 3 h with maximal DNA oxidation at 6 h [42]; the slower effect of 2-NP might again be explained by the requirement of metabolic activation.

In the adult female group, 2-NP did not induce 8-oxodG at all. A sex difference in oxidative damage after treatment of Sprague-Dawley rats with 2-nitropropane has previously been described [39]. The less efficient induction of 8-oxodG in the female rats was explained by a slower metabolism of 2-NP or a more effective protection against oxidative damage in the female rats. This was supported by a study showing differential expression of rat sulfotransferases (i.e. the 2NP metabolising enzyme) between male and female rats [41]. However, no further studies describing this issue, or describing 8-oxodG in female rats after 2-NP treatment have been found.

It is notable that the incision activity in the female group showed the same biphasic pattern as in the male group despite a complete insensitivity to 2-NP in terms of 8-oxodG. As 2-NP also induces 8-aminodeoxyguanosine (8-aminodG) in rat liver tissue [38], the increase in incision activity from 5 to 24 h could perhaps be induced by this base modification. However, it has not been investigated whether 8-aminodG is a substrate for OGG1, although it has been shown that 8-nitroguanine is not a substrate of OGG1 [43,44]. Another explanation is that the levels of the substrate in DNA do not directly determine the regulation of base excision repair of 8-oxodG. A direct effect of 2-NP on the repair mechanisms or a change in redox state and thereby initiation of a signal transduction pathway [45] could also be possibilities.

This study has verified that rapidly dividing foetal tissue exhibits an increased incision capacity to oxidative DNA damage in form of 8-oxodG as compared to adult rats. In accordance higher levels of rOGG1 mRNA were detected. Why this increased repair capacity is not reflected in the levels of 8-oxodG was not clarified. Further investigations in this area will hopefully reveal the regulating mechanisms of 8-oxodG and OGG1 activity in vivo.

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