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Use of repair endonucleases for characterization of DNA damage induced by N-heterocyclic aromatic hydrocarbons★✪

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Several repair endonucleases were used to characterize and quantify various types of DNA damage induced by 7*H*-dibenzo[*c,g*]carbazole (DBC) and its methyl derivative, *N*-methyldibenzo[*c,g*]carbazole (MeDBC). Differences in the DNA damage profile induced by these two derivatives were found to be related to their chemical structure and dependent on the way of their metabolic activation. Different ways of activation gave rise to different numbers of single strand breaks and DNA modifications or, at least, to different ratios of common modifications. DBC induced the highest level of breaks in human hepatal cell line Hep G2, while MeDBC induced most of the breaks in V79 cell line with stable expression of human cytochrome P4501A1. Our results support the idea of two different pathways of biotransformation of DBC and MeDBC.

7*H*-Dibenzo[*c,g*]carbazole (DBC) and *N*-methyldibenzo[*c,g*]carbazole (MeDBC) belong to the group of N-heterocyclic aromatic hydrocarbons (NHA). NHAs represent a relatively minor fraction of crude polycyclic mixtures produced by incomplete combustion of organic material [1–4]; however, these compounds may be equally important carcino-

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Abbreviations: BaP, benzo[a]pyrene; BSA, bovine serum albumine; DBC, 7*H*-dibenzo[*c,g*]carbazole; FaPy, formamidopyrimidine; MeDBC, *N*-methyldibenzo[*c,g*]carbazole; MEM, minimal essential medium; NHA, N-heterocyclic aromatic hydrocarbons; PAH, polycyclic aromatic hydrocarbon.

genic pollutants as PAHs (polycyclic aromatic hydrocarbon) through their own intrinsic activity [5, 6]. Like many other chemical carcinogens, DBC and MeDBC require metabolic activation to electrophilic species before they can interact with DNA and other macromolecules and exert their mutagenic and carcinogenic effects. The microsomal cytochromes P450 catalyze the utilization of oxygen which generates formation of potentially toxic reactive intermediates which bind covalently to DNA, forming DNA-adducts, or induce oxidative DNA damage. Two ways of biotransformation of DBC have been suggested: i) at its ring-carbon atoms, as with PAH, and ii) at the nitrogen position [7–10]. It is supposed that the heterocyclic nitrogen strongly affects the biological activity of DBC and plays an important role in hepatocarcinogenicity. This suggestion is supported by the fact that the *N*-methyl derivative of DBC, MeDBC, lacks the hepatocarcinogenic potential [11] and by the observations that the *O*- and *S*- isoesters of DBC, dinaphtho(2,1,1′,2′)furan and dinaphtho(2,1,1′,2′)thiophene, respectively, lack carcinogenic activity altogether [12].

The aim of this study was to characterize the DNA damage induced by these two derivatives and to identify possible differences in the type of damage in relation to their chemical structure and in dependence on the way of metabolic activation. Different ways were used for activation of DBC and MeDBC: i) subcellular S9 fraction; ii) V79 cell line with stable expression of cDNA of human cytochrome P4501A1; iii) human hepatal cell line Hep G2. In order to characterize DNA damage induced by DBC and its methyl derivative MeDBC, a set of repair endonucleases, which specifically recognize certain DNA base modifications and sites of base loss (AP sites) as well as DNA repair inhibitors were used. For detection of DNA damage induced by DBC and MeDBC a modified alkaline single cell gel electrophoresis has been applied [13]. Benzo[a]pyrene (BaP), the polycyclic aromatic hydrocarbon, was used as a positive control.

MATERIAL AND METHODS

Cell lines. Diploid human fibroblasts VH10; V79 cell line with stable expression of human cytochrome P4501A1 (V79 h1A1) (48 pmol/min per mg total protein); human hepatal cell line Hep G2.

Treatment of cells. Cells, 24 h after inoculation were treated: i) for 2 h with MEM without serum in the presence or absence of S9 fraction and DNA repair inhibitors; ii) for 24 h with complete MEM.

Single cell gel electrophoresis (SCGE). The procedure of Singh *et al.* [14] modified by Collins *et al.* [13] was used. Cells were lysed for 60 min at 4°C (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10.0), washed and incubated with different repair endonucleases: endo III (45 min) and FaPy (30 min) in 40 mM Hepes/KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA (pH 8.0); exo III (30 min) in 36 mM Tris/HCl, 18 mM $CaCl_2$, 0.5 mg/ml BSA (pH 8.0); UV endo (15 min) in 20 mM Tris/HCl (pH 7.5), 100 mM NaCl, 15 mM EDTA at 37°C. The incubation was followed by 40 min of DNA unwinding time and 30 min electrophoresis (0.3 M NaOH, 1 mM Na_2EDTA, 4°C). After neutralization slides were stained with 20 μl of ethidium bromide (10 μg/ml). Comets were examined using an image analysis system (Komet 3.0, Kinetic Imaging Ltd.) or scored visually according to Collins *et al.* [15]. The data were analysed statistically by Student's *t*-test. Endonuclease III (endo III) and formamidopyrimidine-DNA glycosylase (FaPy) were obtained from Dr. A.R. Collins (Aberdeen). UV endonuclease (UV endo) was partially purified from *Micrococcus lysodeicticus* and was provided by Dr. J.E. Cleaver (San Francisco). Exonuclease III (exo III) was purchased from Amersham.

RESULTS AND DISCUSSION

DNA strand breakage represents only one type of lesion caused by genotoxic agents; however, since strand breaks are so readily detected, their measurement has become a standard method of expressing DNA damage. DBC induced a relatively low level of single strand breaks (Figs. 1A, 2A and 3) in comparison with the level of DNA adducts induced *in vitro* or *in vivo* [16–18]. The level of DNA strand breaks was significantly increased when DNA repair inhibitors (hydroxyurea (HU, 2×10^{-3} M) and 1-(β-D-arabinofuranosyl)cytosine (araC, 2×10^{-5} M)) were present during 2 h treatment (Fig. 1A). These results suggested that DBC probably induces two types of DNA damage; unstable damage that is removed very quickly, even during the treatment, and stable damage that persists in DNA for a long period and is detected as DNA adducts. The major products of DBC metabolism are phenols but not dihydrodiols [7, 8, 10, 19, 20]. This phenomenon implies that either the intermediates formed undergo intramolecular rearrangement more readily than PAHs or related NPAs, or that oxidation is exclusively by direct hydroxylation on various carbon atoms. Chen *et al.* [21] have shown that DBC, activated *in vitro* by a microsomal fraction, predominantly forms depurinating adducts by one-electron oxidation; whereas stable unidentified adducts constitute a minor fraction. MeDBC induced approximately the same level of single strand breaks as DBC; however, the level of breaks was not significantly increased in the presence of DNA repair inhibitors (Fig. 1B). The substitution of methyl group at the nitrogen position in MeDBC leads probably to formation of a different spectrum of DNA adducts, that are more stable that those formed by DBC. Marked differences in DNA adducts

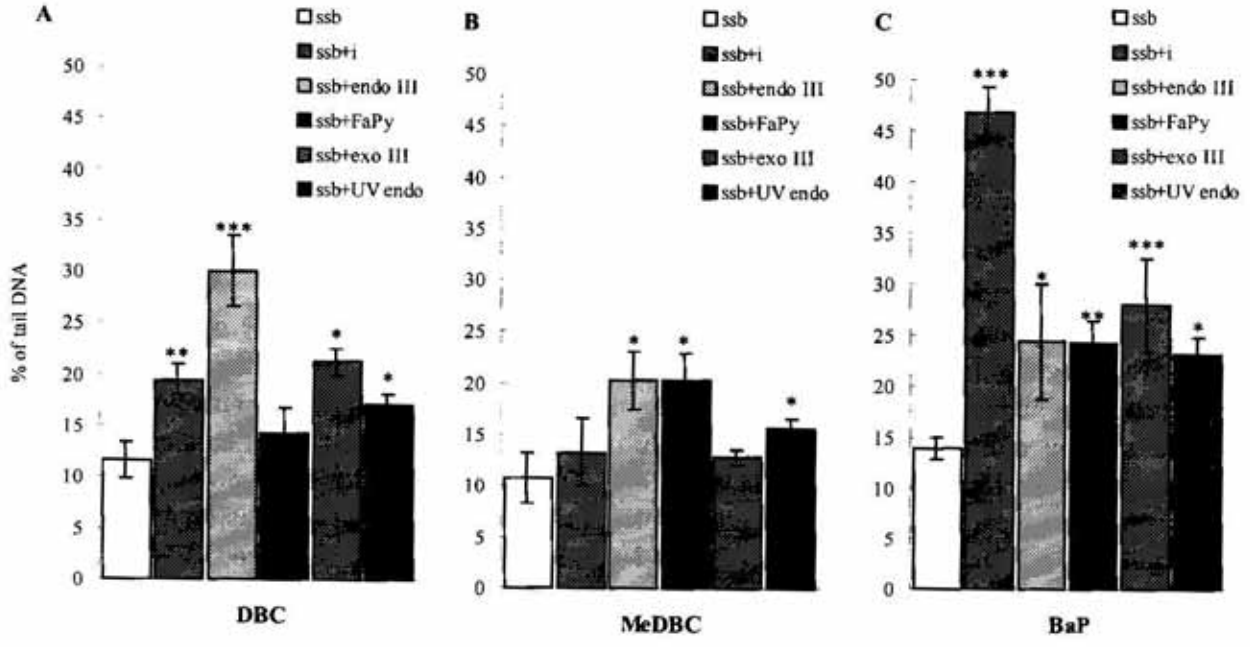

Figure 1. DNA damage profile induced by DBC (A), MeDBC (B), and BaP (C) in VH10 cells after 2 h treatment in the presence of S9 fraction.

VH10 cells were treated with DBC (1 μM), MeDBC (1 μM), BaP (10 μg/ml) in a medium without serum. After lysis of cells in agarose, the nuclei were treated with endo III (45 min), FaPy (30), exo III (30 min), or UV endo (15 min) before being subjected to unwinding and electrophoresis. DNA single strand breaks formed in the presence of repair inhibitors are marked ssb+i. Comets were analyzed using an image analysis system. Statistically different from control: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

patterns elicited by DBC and MeDBC were found *in vivo* [11] and *in vitro* [16, 22] using the ^{32}P-postlabeling method. The level of single strand breaks induced by BaP was significantly increased in the presence of DNA repair inhibitors (Fig. 1C) in contrast to the level induced by MeDBC.

In order to characterize the DNA modifications induced by DBC and MeDBC, a set of repair DNA endonucleases differing in substrate specificity was used [23]. The DNA damage profile obtained by use of different repair endonucleases indicates the relative frequencies of various types of modifications and can serve as a fingerprint of the ultimate DNA damaging agent. For detection of base modifications DNA glycosylases with associated AP lyase activity, endonuclease III, FaPy-DNA glycosylase, and UV endonuclease were used. Endonuclease III (thymine glycol-DNA glycosylase, TG-DNA glycosylase) recognizes pyrimidine residues damaged by ring saturation, ring fragmentation, or ring contraction and regular abasic sites, while formamidopyrimidine-DNA glycosylase (FaPy-DNA glycosylase or FPG protein) releases fragmented and oxidized purines and incises abasic sites. UV endonuclease from *M. lysodeicticus* (pyrimidine dimer-DNA glycosylase, PD-DNA glycosylase) incises selectively DNA at sites of pyrimidine dimers and regular abasic sites. In the absence of pyrimidine dimers only the AP sites are recognized by UV endonuclease. The recognition of abasic sites by FaPy-DNA glycosylase and by endonuclease III is probably similar to that by UV endonuclease. Therefore the number of base modifications recognized by a particular glycosylase can be estimated as a difference between the number of base modifications recognized by FaPy-DNA glycosylase or by endonuclease III, and the number of AP sites recognized by UV endonuclease. The major physiological role of exonuclease III, a multifunctional enzyme, is the cleavage of DNA 5′ adjacent to an AP site. It seems that incision at abasic sites is not the sole function of this enzyme. There are reports of direct 5′ cleavage of DNA containing a base fragment [24] or *p*-benzoquinone bulky adduct [25] without prior generation of an AP site.

Different ways of activation of DBC and MeDBC gave rise to different DNA modifications or at least to different ratios of common modifications. The DNA damage profile induced by DBC after 2 h treatment of VH10 cells in the presence of S9 fraction was dominated by base modifications sensitive to endonuclease III, while base modifications sensitive to FaPy glycosylase were absent. AP sites, recognized by UV endonuclease represented only minor lesions (Fig. 1A). The higher level of breaks detected with exonuclease III than with UV endonuclease could be, perhaps related to the broader spectrum of damage recognized by this enzyme. The DNA damage profile induced by MeDBC, under the same conditions of treatment, consisted of approximately equal levels of base modifications sensitive to endonuclease III and to FaPy glycosylase, while AP sites recognized by UV endonuclease were minor lesions (Fig. 1B). No DNA modifications recognized by exonuclease III were detected. BaP induced approximately equal levels of base modifications sensitive to endonuclease III and FaPy glycosylase, AP sites recognized by UV endonuclease and DNA modifications sensitive to exonuclease III (Fig. 1C).

It has been shown that cytochrome P4501A1 takes part in the metabolism of DBC and its derivatives with sarcomagenic activity [16]. In order to check the role of this cytochrome in DBC and MeDBC biotransformation, the V79 cell line with stable expression of human cytochrome P4501A1 was used. V79 cells which express cytochromes are thought to be a valuable analytical tool for studying CYP-mediated metabolism of xenobiotics in general, and of PAHs or NHAs in particular [26]. Activation of DBC and MeDBC mediated by cytochrome P4501A1 led to different levels of breaks and ratios of DNA modifications. Both DBC and MeDBC increased significantly the level of single strand breaks in V79h1A1 cells

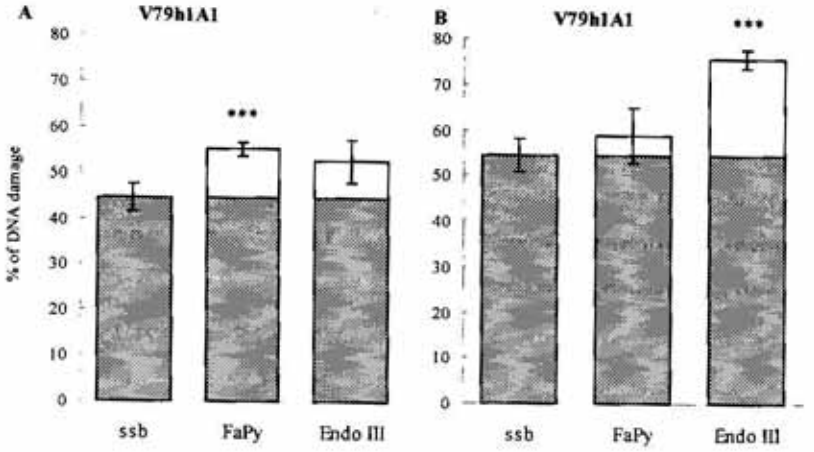

Figure 2. DNA damage profile induced by DBC (A) and MeDBC (B) in V79h1A1 cell line after 24 h of treatment.

V79 cells were treated in complete medium with DBC (1 μM) or MeDBC (1 μM). After lysis of cells in agarose, the nuclei were incubated with endo III (45 min) or FaPy (30 min) before being subjected to DNA unwinding and electrophoresis. Comets were scored visually according to Collins *et al.* [15]. One hundred comets on each slide were scored visually as belonging to one of five classes according to tail intensity and given a value of 0, 1, 2, 3 or 4 (from undamaged 0, to maximally 4). Thus, the total score for 100 comets could range from 0 (all undamaged, 0% of damage) to 400 (all maximally damaged, 100% of damage). Statistically different from control: $^{***}P < 0.001$.

after 24 h of treatment (Fig. 2A and 2B). DBC induced less of strand breaks in V79h1A1 cells than did MeDBC and approximately equal levels of base modifications recognized by endonuclease III and FaPy glycosylase (Fig. 2A). In the DNA damage profile induced by MeDBC in V79h1A1 cells predominated the base modifications sensitive to endonuclease III, whereas the level of base modifications recognized by FaPy glycosylase was negligible (Fig. 2B). These data support the role of cytochrome P4501A1 in biotransformation at the ring-carbon atoms of DBC and its derivatives with sarcomagenic activity.

Marked differences in the level of DNA strand breaks induced by DBC and MeDBC were found in human hepatal cell line Hep G2. DBC induced a significantly higher level of DNA strand breaks in comparison with MeDBC and BaP (Fig. 3). Predominant activity of DBC in Hep G2 cells in comparison with VH10 cells could be, perhaps, connected with high reactivity of the ultimate metabolite of DBC, which requires close contact between the target molecule and activating system. The highest activity of DBC in Hep G2 is in a good correlation with strong hepatocarcinogenic activity of DBC *in vivo*. MeDBC was, in contrast to DBC, more effective in V79h1A1

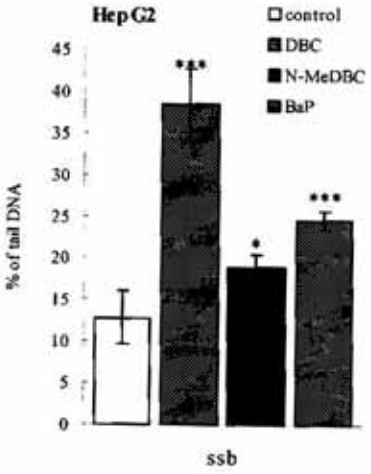

Figure 3. The level of single strand breaks induced by DBC, MeDBC, and BaP in Hep G2 cells after 24 h of treatment.

Hep G2 cells were treated in complete medium with DBC (1 μM), MeDBC (1 μM), BaP (10 μg/ml). Comets were examined using an image analysis system. Statistically different from control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cells. Substitution of methyl group at nitrogen position leads probably to activation of the molecule predominantly at the ring-carbons,

as is the case with PAH. Our results confirm the differences in biotransformation of DBC and MeDBC related to their chemical structure.

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