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Association between the Levels of Biogenic Amines and Superoxide Anion Production in Brain Regions of Rats after Subchronic Exposure to TCDD

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Abstract

The effects of TCDD on the distribution of biogenic amines and production of superoxide anion (SA) in different brain regions of rats have been studied after subchronic exposure. Groups of females Sprague-Dawley rats were administered daily dose of 46 ng TCDD/kg/day (treated groups), or the vehicle used to dissolve TCDD (control group), for 90 days. The rats were sacrificed at the end of the exposure period and their brains were dissected into different regions including, hippocampus (H), cerebral cortex (Cc), cerebellum (C), and brain stem (Bs). The levels of different biogenic amines and some of their metabolites, including, nor epinephrine (NE), dopamine (DA), 3,4-dihydroxy phenyl acetic acid (DOPAC), 4-hydroxy,3-methoxy-phenyl acetic acid (HVA), 5-hydroxy tryptamine (5-HT), and 5-hydroxy indole 3-acetic acid (5-HIAA), were determined in those brain regions, using a High Performance Liquid Chromatography (HPLC) system with an electrochemical detector. SA production was also determined in those regions, using the cytochrome c reduction method. Results of analyses indicate significant increases in the levels of DA, NE and DOPAC in H, NE and HVA in Cc, NE and DA in Bs and NE in C. SA production was significantly increased in H and Cc, but not in Bs or C. The results also indicated strong correlations between DA and DOPAC, and SA and NE in all of the brain regions, and also between SA and 5-HT/HIAA in H and Cc. These results may indicate the contribution of biogenic amines, especially NE and 5-HT/HIAA to SA overproduction in some brain regions and may also indicate the potential of long term neurotoxic effects of those biogenic amines, in response to subchronic exposure to TCDD.

Keywords

TCDD; Brain; Cerebellum; Brain stem; Cerebral cortex; Hippocampus; Subchronic toxicity; superoxide anion; Biogenic amines; HPLC

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a persistent environmental contaminant that produces a wide range of acute and long term toxic, and biochemical effects in experimental animals (Alsharif *et al.*, 1999; DeVito and Birnbaum, 1994; DeVito *et al.*, 1997; Hunter *et al.*, 1999; Kimbrough, 1974; Kociba *et al.*, 1976; 1978; Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982; Safe, 1990). One of the most prominent acute effects in rats, is hypohagia or

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feed intake reduction that can lead to the death of animals (Rozman, *et al.*, 1991; Stahl *et al.*, 1991; Tuomisto *et al.*, 1990; Unkila *et al.*, 1993; 1995; 1999). In an effort to understand the mechanism of hypophagia, studies were focused on determining the effects of acute doses of TCDD on the levels of biogenic amines in the brains of the animals (Rozman *et al.*, 1991; Tuomisto *et al.*, 1990; Unkila, *et al.*, 1993). Biogenic amines are a class of low-molecular weight transmitters that includes the catecholamines, such as dopamine (DA) norepinephrine (NE) and epinephrine (E), and the serotonin or 5-hydroxytryptamin (5-HT), which are synthesized in brain. DA is synthesized from tyrosine, and about half of the DA formed in the cytoplasm is actively transported into DA beta hydroxylase-containing storage vesicles, where it is converted to NE. The remainder is deaminated to 3,4-Dihydroxyphenylacetic acid (DOPAC), and subsequently o-methylated to 4-hydroxy-3-methoxy-phenylacetic acid (HVA) by monoamine oxidase (MAO) and catechol o-methyl transferase (COMT), respectively. 5-HT is synthesized from tryptophan, and could be degraded by MAO, giving rise to 5-hydroxyindole-3 acetic acid (5-HIAA). Rozman *et al.* 1991 have observed progressive, and time-dependent increases in tryptophan levels in plasma and brain of TCDD-treated rats, which were paralleled by increases in brain 5-HT and 5-HIAA, and have suggested a serotonergic mechanism for TCDD-induced hypophagia. However, studies by Tuomisto *et al.* (1990) have demonstrated minor changes in brain neurotransmitter systems of rats, in response to lethal doses of TCDD, and suggested that the observed changes are not likely the key mediators of TCDD-induced hypophagia. Similarly, Unkila *et al.* 1993 argued against a crucial role for biogenic amines as mediators of TCDD-induced hypophagia, although they found that TCDD increased tryptophan level in some brain regions of rats. Also, Stahl *et al.* (1991) have shown that depletion of brain serotonin does not alter TCDD-induced hypophagia in rats.

Studies on the tissue distribution of TCDD in mice have found significant and dose-related recovery of the compound in different tissues, including the brain (Diliberto, *et al.*, 1995). Long-term exposure of rodents to TCDD was found to result in dose-dependent increases in the production of reactive oxygen species (ROS), lipid peroxidation (LP) and DNA damage in brain tissues (Hassoun *et al.*, 1998, 2000, 2002). Studies on the long term TCDD effects on various brain regions of rats have demonstrated significant increases in different biomarkers of oxidative stress, associated with suppression of different antioxidant enzyme activities in the cerebral cortex (Cc) and hippocampus (H), but not in the cerebellum (C) or brain stem (BS) (Hassoun *et al.*, 2003).

Oxidative deamination of biogenic amines by the mitochondrial and cytosolic MAO is found to be associated with production of large concentrations of ROS (Agostinelli, *et al.*, 2004; Cadenas and Davies, 2000; Seraki and O'Brien, 2002; Toninello *et al.*, 2004). Catecholamines were also found to cause a reduction in intracellular glutathione level and accumulation of ROS in oligodendrocytes, in culture (Korchid *et al.*, 2002). Incubation of some DA precursors and related catechols with DNA, proteins and lipids was shown to result in oxidative damage to these molecules, the majority of which appeared to be mediated by ROS, such as superoxide anion (SA) and hydroxyl radicals, in addition to semiquinone radicals and quinones (Pattison, *et al.* 2002).

This study was undertaken to investigate the long term effect of TCDD exposure on the levels of some biogenic amines and their metabolites in different brain regions of rats, and also the possible association between those changes and TCDD-induced production of ROS, in select brain regions of those rats.

MATERIALS AND METHODS

Chemicals

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was purchased from the National Cancer Institute (NCI) chemical repository (Kansas City, MO) and was 98% pure. All other chemicals used for various assays were obtained from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade or of the highest grade available.

Animals and treatments

Harlan Sprague-Dawley female rats weighing 170–190 g were used for this study. The animals were 8 weeks of age at the time of first exposure and were given pelleted diet from Harlan-Teklad (Indianapolis, Indiana), and tap water *ad libitum*. TCDD was administered by oral gavage to groups of rats (12 rats/group), at a dose of 46 ng/kg/day, for 13 weeks. The dosing regimen of TCDD was based on previous studies to produce significant oxidative stress in select brain regions (Hassoun *et al.*, 2003;2004). Control groups were given the vehicle used to dissolve TCDD (1% acetone in corn oil), and the volume rate of administration was kept at 2.5 ml/kg body weight. At the end of week 13 of treatment, the animals were euthanized using carbon dioxide asphyxiation, followed by decapitation, and brains were immediately removed and dissected over ice-cold glass slides to various regions including, cerebral cortex (Cc), hippocampus (H), cerebellum (C), and brain stem (Bs). Four pooled samples of each region (3 animals/pooled sample), were used. Pooled tissues were divided in to two portions, one was used for the determination of SA production and the other for the determination of the levels of biogenic amines.

Determination of the production of superoxide anion (SA)

SA is one form of damaging ROS, and is a precursor for short lived, tissue damaging oxygen free radicals, such as the hydroxyl radical (Davies, 1995). Production of SA in brain tissue homogenates was measured according to the method of Babior, *et al.* 1973, which is based on the reduction of cytochrome c. Brain tissues were homogenized in Tris-KCl buffer (0.05 M Tris and 1.15% KCl, pH 7.4), using a Potter-Elvehjem homogenizer fitted with Teflon pestle, to produce a 10% homogenates. The 2 ml reaction mixtures contained 25 μ l of the homogenate and 0.05 mM cytochrome c in Tris-KCl buffer, pH 7.4. The reaction mixtures were incubated for 15 minutes at 37°C, after which, reactions were terminated by placing the tubes on ice. The mixtures were centrifuged at 700 $\times$ g for 10 minutes, and the supernatant fractions were collected for subsequent spectrophotometric measurement at 550 nm, using a Spectronic-20 spectrophotometer. Absorbance values were converted to nmoles of cytochrome c reduced/minute, using the extinction coefficient $2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Babior *et al.*, 1973).

Standard and sample Preparation for HPLC analysis

Tissue samples were prepared and analyzed for biogenic amines and their metabolites, using reverse phase High Performance Liquid Chromatography System (HPLC), with an electrochemical detector, as previously described (Cheng and Kuo, 95; Liu *et al.* 2000; Chi *et al.*, 99; Auger *et al.*, 2000). The apparatus used consisted of Waters pumps (Waters 600E Multisolvant Delivery System), 600 Controller, 717 Plus Autosampler and a 464 Pulsed Electrochemical detector (Waters, Milford, MA), and was controlled by a Dell computer using Mellenium³² software. Chemical constituents were separated utilizing a C18 μ Bondapack (3.9 $\times$ 150mm) (Waters, Milford, MA). The mobile phase consisted of 50 mM phosphatate buffer (pH= 3.5) containing 80 mg/L octanesulfonic acid (OSA), 25 mg/L ethylenediaminetetraacetic acid (EDTA), and 10% v/v methanol. Na-HPO₄.H₂O, EDTA, o-phosphoric acid and OSA were all dissolved in HPLC grade water and then passed through a 0.45 μ m filter and degassed under a vacuum. The mobile phase was prepared daily and was run overnight for use in the following

day. In all instances (standard and sample analysis), the mobile phase flow rate was 1.5 ml/min. The electrochemical detector consisted of a glassy carbon working electrode and an Ag/AgCl reference electrode. The applied oxidation potential was set at 750 mV. The samples were run under two different scales: 100 nA and 10 nA full scale. The reasoning for the two different scales was because of the magnitude of the response elicited by NE relative to the other analytes contained in the sample. This required NE to be analyzed with the detector set to 100 nA corresponding to full scale deflection while the remaining peaks could be quantified more accurately at 10 nA corresponding to a full scale deflection. Three standard mixtures were prepared daily in 0.1 N perchloric acid, which contained NE, DA, DOPAC, 5-HIAA, 5-HT, HVA, and the internal standard 3,4-dihydroxybenzyl amine hydrobromide (DHBA), at the following concentrations (ng/ml), respectively, 4.9, 10.35, 6.8, 5.9, 6.55, 8.75, 28.9, and 5.5 (mixture I), 19.6, 41.4, 22, 23.6, 26.2, 35, 115.6, and 27.2 (mixture II), 78.4, 165.6, 88, 94.8, 104.8, 140, 462.4, and 108.8 (mixture III). The relative amounts of each biogenic amine in the above mixtures were chosen such that the peak heights for each of the compounds in the mixture were equivalent. The standard curve was run at 10 and 100 nA corresponding to full scale to account for intra-day variation in peak retention time and response of the working electrode.

Pooled tissue samples (75 mg) were homogenized in a disposable polypropylene microtube with pestle (Fisher Scientific, Hanover Park, IL), containing 150 μ l of ice-cold, 0.1 N perchloric acid, for 30 seconds. The samples were then centrifuged at $16060 \times g$ for 30 minutes at 4° C. 100 μ l of the supernatant was removed, and 10 μ l of DHBA was added as an internal standard. The 110 μ l mixture was then filtered, using a 0.2 μ m filter (Corning, NY), and 10 μ l of that mixture was immediately analyzed by HPLC, as indicated above.

Protein determination

The amounts of protein were determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Data analyses and statistical methods

Data for the biogenic amines are expressed as the mean of 4 pooled samples (3 animals/pooled sample) $\pm$ the standard error of the mean (SEM). Data were subjected to a two sample, assuming equal variance t-test. Data for SA production are expressed as the mean of 4 pooled samples (3 animals/pooled sample) $\pm$ standard deviation (SD), and were analyzed, using a t-test. A significance level of $p < 0.05$ was employed for all analyses. Pearson's correlation coefficients were calculated using Microsoft Excel.

RESULTS

Figure 1 demonstrates the effects of treatment of rats with 46 ng TCDD/kg/day on the production of SA in different brain regions, as compared with their corresponding controls. Significant levels of SA production were seen in H and Cc, but not in the C or Bs of the TCDD-treated rats.

Figure 2 shows the distribution levels of DA in various brain regions for control, and TCDD-treated rats. TCDD treatment resulted in significant increases in the levels of DA in H and Bs, but not in the other brain regions, as compared with the corresponding regions of the control group.

The distribution levels of NE in the four brain regions of control and TCDD-treated rats are shown in figure 3. While NE could not be detected in any of the brain regions of the control rats, significant levels could be detected in the four brain regions of the TCDD treated rats.

The effects of TCDD treatment on the distribution of the DA metabolite, DOPAC in various brain regions of rats are shown in figure 4. Treatment of rats with TCDD resulted in a significant increase in the level of DOPAC in the H, with no significant changes observed in the other regions, as compared with the corresponding regions of the control.

Subsequent metabolism of DOPAC by COMT results in production of HVA. The effects of TCDD treatment on the distribution of HVA in various brain regions are demonstrated in figure 5. While HVA could not be detected at any level in the Cc, Bs and C of the control group, significant level of the compound was detected in the H of those animals. Treatment with TCDD resulted in the detection of a significant level of HVA in the Cc, with no significant changes observed in any other brain regions, as compared with their corresponding controls.

Figure 6 shows the effects of TCDD treatment on the distribution levels of 5-HT and its metabolite 5-HIAA, in different brain regions of rats. The two compounds were added together, because when running the tissue samples the peaks could not be always separated effectively. This was due to the fact that these two peaks eluted relatively closely to one another and could not be adequately separated without adversely affecting the separation of peaks with shorter elution times. In retrospect, it would have been ideal to have larger amount of sample available so that each individual sample could be run under various chromatographic conditions in order to adequately separate all peaks of interest. While the compounds could be detected in H, Cc and the Bs of the control, they could not be detected at any level in the C of the same rats. TCDD administration resulted in the detection of those compounds in the C, and a significant increase in the levels of the compounds in Bs. However, the treatment did not result in significant changes in the levels of the compounds in the H and Cc, as compared with their corresponding controls.

Table 1 shows the correlations between the levels of DA and NE and DA and DOPAC in different brain regions. Because HVA could not be detected in most of the brain regions of the control, correlations between the level of this metabolite and DA or DOPAC could not be calculated. Table 1 also shows the correlations between SA production in various brain regions of rats treated with TCDD, and the levels of different biogenic amines in those same regions. The values presented in the table are Pearson's correlation coefficients, and values approximating 1.0 are considered strong with negative signs indicating inverse correlations between the studied markers. While weak correlations between DA and NE were found in the H and Cc, relatively stronger correlations were observed between the two biogenic amines in the Bs and C. The table also shows strong correlations between DA and its metabolite DOPAC, in all of the brain regions. Strong correlations between SA and NE levels were revealed in H and Cc, with relatively weaker correlations revealed in C and Bs regions, respectively. Relatively less strong correlations were also observed between SA and DA levels as well as between SA and HVA in Cc region, and between SA and 5-HT/5-HIAA in H and Cc regions.

DISCUSSION

Due to the environmental persistence of TCDD and its long half life in animals and humans (Safe, 1990; Van Den Berg *et al.*, 1998), studies on the potential long term effects of the compound are important. In this study, subchronic exposure of rats to low doses of TCDD is shown to produce larger changes in the levels of some biogenic amines and their metabolites in the brain, than the previously observed after acute exposure (Tuomisto, *et al.* 1990). Since changes in the levels of biogenic amines are known to be associated with various human diseases (D' Amato *et al.*, 1987; Hastings and Zigmond, 1997; Horneykiewicz, 1975; Okado *et al.*, 2001; Richelson, 1991; Tohgi, *et al.*, 1997), these results may indicate possible long term neurotoxic effects of TCDD.

Previous studies (Hassoun *et al.*, 2003,2004), as confirmed by the results of the present study, have indicated significant induction of oxidative stress in select brain regions of rats after long term exposure to TCDD. The results also indicate association between induction of oxidative stress and changes in the levels of different biogenic amines and/or their metabolites in those regions. These results are in agreement with previous studies showing association between different biogenic amines and ROS production in neuronal tissues (Agostinelli, *et al.*, 2004;Naoi, *et al.*, 2005;Siraki and O'Brien, 2002;Cadenas and Davies; 2000), and further indicate the contribution of TCDD exposure to those changes

While NE could not be detected in the brain regions of the control animals, significant levels have been detected in those of the TCDD treated rats. Studies by Milosheva *et al.* (2003) have shown that NE is released under energy deprivation, in rat hippocampal slices. Also, administration of low doses of TCDD to guinea pigs, was shown to cause profound reduction of glucose uptake by adipose tissue, pancreas and brain (Enan *et al.*, 1992). Therefore it is likely that NE was released at detectable levels in different brain regions, in response to energy deprivation caused by TCDD.

The results show weak correlations between DA (the precursor for NE synthesis) and NE levels in different brain regions. This suggests that TCDD did not affect the rate of NE synthesis, but rather affected the release of the biogenic amine from the stores.

Some of the synthesized DA is metabolized to DOPAC, which can be further converted to HVA, and these reactions are carried out by MAO and COMT, respectively (Lefkowitz, *et al.*, 1996). This may explain the strong correlations between DA and DOPAC levels in different brain regions, i.e, the levels of metabolites are positively correlated with DA levels in those regions. The reason for the detection of higher levels of DOPAC and HVA in H and Cc, respectively, could be due to decreases in the rate of outward transport of metabolites across the membranes in those regions. Previous studies have shown that treatment with a similar TCDD regimen produces significant production of lipid peroxidation in H and Cc (Hassoun *et al.*, 2003). Knowing that lipid peroxidation can alter the functions of different cellular membranes (Davies, 1995), changes in the transport of different molecules across the membranes are possible.

The results of the study revealed strong correlations between SA and NE in the H and Cc regions, with weaker correlations revealed between the same biogenic amine and SA in the C and Bs regions. Also, significant levels of NE have been detected in all of the brain regions. With regard to DA, strong correlations were found between this biogenic amine and its metabolite DOPAC, in different brain regions, with weak correlations revealed between the same biogenic amine and its metabolites and SA. This may indicate a minor contribution of DA and its metabolites to the observed SA over production in select brain regions. Conversely, the observations may indicate the major role of NE to the observed overproduction of SA in H and Cc, possibly through the process of metabolism. Further studies to determine the rate of NE metabolism in those regions are necessary to confirm that. The correlations also confirm the conclusion of previous studies indicating protection of the C and Bs regions against TCDD-induced oxidative stress. However, they do not exclude the effects that may be produced by increased levels of NE in those two particular regions, as well as in the H and Cc regions. Similarly, the effects of increased levels of DA in the H and Bs regions should not be excluded, since significantly higher levels of this biogenic amine were detected in those two regions, in response to TCDD.

Relatively strong correlations between SA and 5-HT/5-HIAA in the H and Cc regions, but not in the Bs or the C region were observed. However, significant levels of 5-HT/5-HIAA have been reported in the Bs and C regions, but not in the H or Cc regions, in response to TCDD

treatment. knowing that 5-HT metabolism is associated with 5-HIAA, as well as ROS production, and strong correlations between SA and 5-HT/5-HIAA were revealed in H and Cc, the measured levels in H and Cc regions could be solely contributed to 5-HIAA. Conversely, the measured amounts in the C and Bs regions could have been contributed to 5-HT, rather than 5-HIAA. These observations also indicate that the Bs and C regions may not be protected against TCDD-induced increases in 5-HT levels, although they were found to be protected against TCDD-induced oxidative stress.

Although the correlations between SA and 5-HT/5-HIAA in the H and Cc regions are weaker than those observed between NE and SA in those same regions, they are still interesting and may indicate possible contribution of the biogenic amine and/or its metabolite to the induction of oxidative stress in those particular regions. Siraki and O'Brien (2002) have found that the order of prooxidant activity of neurotransmitter phenols and hydroxyindoles in catalyzing beta-NADH or cystein cooxidation, when metabolically activated by peroxidase/H₂O₂ was, tyramine> acetyltyrosine>tyrosine>serotonin (5-HT)>n-acetylserotonin, 5-HIAA. Knowing that tyrosine is the key amino acids for DA-NE synthesis (Lefkowitz et al, 1996), this order likely reflects the strength of correlations between the studied biogenic amines and SA, reported in this study.

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Figure 1.

Effects of subchronic treatment with 46 ng TCDD/kg/day on superoxide anion (SA) production in various brain regions. Columns indicated by *, are significantly different ($p < 0.05$) from the corresponding control of a similar brain region, using t-test.

Figure 2.

Effects of subchronic treatment with 46 ng TCDD/kg/day on the distribution level of dopamine (DA) in various brain regions. Columns indicated by *, are significantly different ($p < 0.05$) from the corresponding control of a similar brain region, using a two sample, assuming equal variance t-test.

Figure 3.

Effects of subchronic treatment with 46 ng TCDD/kg/day on the distribution level of nor epinephrine (NE) in various brain regions. Columns indicated by *, are significantly different ($p < 0.05$) from the corresponding control of a similar brain region, using a two sample, assuming equal variance t-test. ND (not detected)

Figure 4. Effects of subchronic treatment with 46 ng TCDD/kg/day on the distribution level of 3,4-Dihydroxyphenylacetic acid (DOPAC) in various brain regions. Columns indicated by *, are significantly different ($p < 0.05$) from the corresponding control of a similar brain region, using a two sample, assuming equal variance t-test.

Figure 5.

Effects of subchronic treatment with 46 ng TCDD/kg/day on the distribution level of 4-hydroxy-3-methoxy-phenylacetic acid (HVA) in various brain regions. Columns indicated by *, are significantly different ($p < 0.05$) from the corresponding control of a similar brain region, using a two sample, assuming equal variance t-test. ND (not detected)

Figure 6.

Effects of subchronic treatment with 46 ng TCDD/kg/day on the distribution level of 5-hydroxy tryptamin (5-HT) and its metabolite 5-hydroxyindole-3 acetic acid (5-HIAA), in various brain regions. Columns indicated by *, are significantly different ($p < 0.05$) from the corresponding control of a similar brain region, using a two sample, assuming equal variance t-test. ND (not detected)

Table 1

Pearson's correlation coefficients between SA and various biogenic amines and between different catecholamines and their metabolites in different brain regions of rats treated with TCDD, subchronically. Data from 4 samples of each biomarker in each brain region were compared with data of a similar sample number for a second biomarker, in the same brain region.

[[Biomarkers studied]] [[I]]	[[H]]	[[Pearson's correlation coefficient]]		
		[[Cc]]	[[Bs]]	[[C]]
[[SA-DA]]	[[−0.02]]	[[0.51]]	[[0.27]]	[[−0.20]]
[[SA-NE]]	[[0.85]]	[[0.89]]	[[0.22]]	[[0.57]]
[[SA-DOPAC]]	[[−0.38]]	[[0.46]]	[[0.25]]	[[−0.35]]
[[SA-HVA]]	[[−0.14]]	[[0.55]]	[[NC]]	[[NC]]
[[SA-5-HT/5-HIAA]]	[[0.62]]	[[0.47]]	[[−0.13]]	[[0.32]]
[[DA-NE]]	[[0.02]]	[[0.16]]	[[0.51]]	[[0.47]]
[[DA-DOPAC]]	[[0.84]]	[[0.97]]	[[0.93]]	[[0.90]]