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# METHYLMERCURY-INDUCED CHANGES IN MITOCHONDRIAL FUNCTION IN STRIATAL SYNAPTOSOMES ARE CALCIUM-DEPENDENT AND ROS-INDEPENDENT

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## Abstract

The brain is the main target organ for methylmercury (MeHg), a highly toxic compound that bioaccumulates in aquatic systems, leading to high exposure in humans who consume large amounts of fish. The mechanisms responsible for MeHg-induced **changes in neuronal function** are, however, not yet fully understood. In the present study we investigated whether MeHg-induced elevations in reactive oxygen species (ROS) or intracellular calcium are responsible for altering mitochondrial metabolic function in rat striatal synaptosomes.

MeHg decreased mitochondrial function (measured by the conversion of MTT to formazan) and increased ROS levels in striatal synaptosomes after 30 min exposure. Although co-incubation with the antioxidant Trolox **significantly** reduced MeHg-induced ROS levels, it failed to restore mitochondrial function. MeHg also increased cytosolic and mitochondrial calcium levels in striatal synaptosomes. These elevations were **largely** independent of extra-synaptosomal calcium, given that nominal calcium free buffer with 20 μM EGTA did not prevent MeHg-induced increases in cytosolic calcium. In conclusion, we suggest that ROS are not the cause of mitochondrial dysfunction **in striatal synaptosomes** after MeHg exposure; rather, we propose that ROS formation is a downstream event that reflects MeHg-induced mitochondrial dysfunction due to increased mitochondrial calcium levels.

### Keywords

Methylmercury; striatal synaptosomes; mitochondria; ROS; calcium

## Introduction

Methylmercury (MeHg) is an environmental contaminant whose primary target is the central nervous system (National Research Council, 2000). The mechanisms for MeHg-induced **neurotoxicity** are still not clearly characterized. However, processes known to be affected by MeHg include alterations in cytoskeletal function, neurotransmitter release and excitotoxicity,

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protein synthesis, calcium homeostasis, mitochondrial dysfunction, formation of reactive oxygen species and free radicals, and induction of cell death by apoptosis or necrosis (for review, see Aschner and Syversen, 2005; Sanfeliu et al., 2003).

Previously, we have shown that MeHg increased ROS levels and decreased both mitochondrial membrane potential and MTT conversion to formazan in rat striatal synaptosomes, with the greatest effects occurring in synaptosomes from younger animals (Dreiem et al., 2005). However, the relationship between ROS formation and mitochondrial damage after MeHg exposure is not fully understood. **ROS** can cause oxidative damage to mitochondria, leading to reduced enzyme activity and compromised mitochondrial function (Galindo et al., 2003, Radi et al., 2002). On the other hand, ROS can also be produced by the mitochondria via leakage of electrons from the electron transport chain to $O_2$, forming superoxide anion radicals ($O_2^{\bullet}$). **Although this** process normally occurs at a low rate in intact respiring mitochondria, $O_2^{\bullet}$ production can increase dramatically if mitochondria are damaged by toxicants (Halliwell and Gutteridge, 1999). Similarly, high cytosolic calcium levels will lead to uptake of calcium to the mitochondria, causing depolarization of the mitochondrial membrane and increased $O_2^{\bullet}$ production (Halliwell and Gutteridge, 1999).

In the present study we examine the relationships between alterations in mitochondrial function, ROS formation, and calcium homeostasis after MeHg exposure. We chose to use striatal synaptosomes, rather than the more commonly used cerebellar granule cell, because of recent data collected in our lab (Dreiem, et al. 2005) demonstrating that MeHg alters both striatal synaptosomal ROS and synaptosomal mitochondrial function. These published data provided the opportunity to begin to investigate the relationship(s) between these two events.

## Materials and Methods

### Materials

Adult (10−14 weeks) Long Evans male rats were obtained from Taconic Farms (Germantown, NY). Methylmercuric chloride (98%) was purchased from EM Sciences (Gibbstown, NJ). Carbonyl cyanide m-chlorophenylhydrazone (CCCP) and methylthiazoletetrazolium (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Trolox was purchased from Calbiochem EMD Biosciences (San Diego, CA). 2’,7’-dichlorofluorescin-diacetate (DCFH-DA), fura-2 acetoxymethyl ester (fura-2 AM), and rhod-2 acetoxymethyl ester (rhod-2 AM) were purchased from Molecular Probes (Eugene, OR). Pierce BCA protein assay reagents were purchased from Pierce Biotechnology, Inc. (Rockford, IL). All other chemicals were analytical grade.

### Preparation of striatal synaptosomes

All procedures involving animals were performed according to the protocols of the Wadsworth Center Institutional Animal Care and Use Committee. Striatal synaptosomes were prepared as described previously (Andersen et al., 2003). Briefly, naïve male Long Evans rats, 10−14 weeks old, were sacrificed, and the brains were removed and placed on ice. The striata were dissected out, pooled, and homogenized in 10 vol 0.32 M sucrose in a Potter-Elvehjem homogenizer, and the homogenate was centrifuged at 4°C for 10 min at 600 × g in a Beckman TJ-6 centrifuge. The supernatant was then diluted 1:1 with 1.3 M sucrose, to yield a suspension at a final concentration of 0.8 M sucrose. This suspension was further centrifuged at 20,000 × g for 30 min at 4°C. The supernatant was discarded, and the pellet, containing synaptosomes and mitochondria, was resuspended in oxygenated HEPES buffer, pH 7.4, at a concentration of approximately 17 μg protein per μl (measured using the Pierce BCA protein assay, with bovine serum albumin as a standard). The **resulting synaptosomes (P2 fraction)** were kept on ice until the experiments were performed, usually within 15−20 min.

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### Assessment of ROS formation

ROS formation was assessed using DCFH-DA, a non-fluorescent cell-permeable compound, that can be oxidized by hydroxyl radicals, peroxynitrite, or hydrogen peroxide (in the presence of peroxidases) to a fluorescent compound, 2',7'-dichlorofluorescein (DCF) (Myhre et al., 2003). Synaptosomes were diluted 1:40 in HEPES buffer before loading with 10 μM DCFH-DA for 15 min at 37°C. DCFH-DA stock solutions (10 mM in dimethylformamide) were made fresh daily. After incubation, the synaptosomes were pelleted by centrifugation (1200 × g for 7 min), and the buffer was replaced with fresh HEPES buffer only.

Working solutions of MeHg were prepared daily by dilution of a stock solution (100 mM MeHg in dimethylformamide [DMF]) in HEPES buffer to 1.67× the desired final concentration. In experiments where the effects of Trolox were studied, the inhibitor was added to the working solution together with MeHg. DMF was added to the working solutions, when necessary, to achieve the same concentration in all samples (final concentration 0.025 %). Working solutions (150 μl) were placed in each well of a 96-well microplate (Costar), and the reaction was started by the addition of 100 μl of the synaptosome solution to each well (final reaction volume 250 μl). Four wells were used for each condition. Each plate was incubated in a shaker incubator at 37°C for 30 min before fluorescence was recorded using a Perkin Elmer LS50 spectrometer (excitation wavelength 488 nm, emission wavelength 525 nm, band widths 5 nm). Blank values were obtained from wells containing buffer and synaptosomes that had not been loaded with DCFH-DA. DCF fluorescence values were corrected for autofluorescence of the samples. The variation between replicate ROS experiments with respect to baseline values was +/− 14.3 fluorescence units, based on a grand mean of means of 104.4 units derived from four separate experiments.

### Assessment of mitochondrial metabolic function

Mitochondrial metabolic function was assessed by the conversion of the dye methylthiazoletetrazolium (MTT) to formazan (Castoldi et al., 2000). Synaptosomes were prepared as described above, and then diluted 1:40 in HEPES buffer. Working solutions of MeHg and inhibitors were made as described above and aliquoted into microtubes (150 μl per tube), and 100 μl of the synaptosome suspension and 25 μl of a 5.0 mg/ml solution of MTT in HEPES buffer were added to each tube. The samples were then incubated for 30 min at 37°C. Formazan crystals were pelleted by centrifugation, dissolved in DMSO, and quantitated spectrophotometrically at 570 nm using an EL808 Ultra Microplate Reader (Bio-Tek Instruments, Inc). Data were expressed as percentage of vehicle control values.

### Chelation of extrasynaptosomal calcium

For studies of the role of extracellular calcium in MeHg-induced ROS formation and mitochondrial dysfunction, synaptosomes were isolated and prepared in normal HEPES with 1.2 mM calcium as described above. Synaptosomes were then pelleted by centrifugation, and resuspended in calcium-free buffer with 20 μM EGTA. For assessment of ROS formation, the synaptosomes were loaded with DCFH-DA in normal buffer with calcium. After centrifugation, the buffer with DCFH-DA was replaced with DCFH-DA-free, calcium-free buffer with 20 μM EGTA This procedure is identical to that described by Voie and Fonnum (2000).

### Cytosolic calcium levels

Cytosolic calcium levels were determined using the ratiometric calcium indicator fura-2. A stock solution of 1 mM of fura-2 AM was made in dimethylsulfoxide and stored at −70°C. Synaptosomes were loaded with 5 μM fura-2 AM in HEPES buffer for 30 min at 37°C, protected from light. After centrifugation, the synaptosomal pellet was resuspended in fresh,

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dye-free HEPES buffer and kept on ice, **protected from light**, until experiments were performed. **Cytosolic** calcium levels were assayed in a Perkin Elmer LS50 spectrometer, with excitation wavelengths alternating between 340 and 380 nm (slit widths 10 nm), and an emission wavelength of 510 nm (slit width 7.5 nm). The assay was performed in a thermostated cuvette (37°C) to which 1.9 ml HEPES buffer and 100 μl synaptosome suspension were added. After allowing for temperature equilibration, baseline data were collected for 60 sec prior to addition of MeHg (100 × final concentration in HEPES buffer). Fluorescence was then measured for 15 min. Untreated controls (buffer only) and vehicle (DMF) controls were included in each replication. For investigation of the role of extracellular calcium, synaptosomes loaded with fura-2 AM in the presence of calcium were spun down and resuspended in calcium-free buffer with EGTA (final conc. 20 μM). MeHg stocks were made in calcium-free buffer with EGTA, and were added as described above.

### Mitochondrial calcium levels

The fluorescent indicator rhod-2 was used to assess mitochondrial calcium levels. In order to improve mitochondrial localization of the indicator (Hajnoczky et al., 1995), we reduced rhod-2 AM to dihydrorhod-2 AM by incubation with sodium borohydride as described in Molecular Probes Product Information **Bulletin** X01244. The synaptosomes were loaded with 5 μM dihydrorhod-2 for 30 min at 37°C before they were pelleted by centrifugation, and the buffer with dihydrorhod-2 was replaced with fresh, dye-free buffer. The synaptosomes were incubated **for an additional** 30 min at 37°C, to allow deesterification of the indicator. The synaptosomes were kept on ice **and protected** from light until the experiments **were** performed. The effects of MeHg on mitochondrial calcium levels were assessed in a Perkin Elmer LS50 spectrometer with excitation wavelength 550 nm and emission wavelength 580 nm, slit widths 7.5 nm. The measurements were performed as described above for the cytosolic calcium measurements with fura-2.

### Statistical analysis

Data were compared for significant differences by analysis of variance (ANOVA), except for calcium measurements, which were analyzed by ANOVA with repeated measures. When the null hypothesis was rejected ($p \leq 0.05$), a *post hoc* test was performed to determine the source of the significant effect(s). Concentration-effect curves were analyzed by Dunnett's test, and the effect of inhibitors was analyzed by Bonferroni test. Differences were considered significant when $p \leq 0.05$.

## Results

### Effects of MeHg on mitochondrial function

MeHg decreased mitochondrial metabolic function, assessed by the conversion of MTT to formazan, in a concentration-dependent manner. Significant reductions in formazan production were seen at MeHg concentrations ≥ 5 μM (Fig. 1). At 10 μM MeHg, MTT conversion to formazan was reduced to 35% of the control value. Hydrogen peroxide ($H_2O_2$), known to be toxic to cells through its induction of oxidative damage (Ricart and Fiszman, 2001), was used as a positive control at 100 μM and reduced formazan production by 50%. The MeHg-induced decrease in formazan production was not altered by shifting to calcium-free HEPES buffer containing 20 μM EGTA before MeHg exposure (Table 1).

### Effects of MeHg on ROS formation

MeHg increased ROS levels in striatal synaptosomes in a concentration-dependent manner (Fig. 2). ROS formation increased linearly with time for at least 60 min. after MeHg addition (data not shown). Significant increases were observed at MeHg concentrations ≥ 5 μM. A

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maximal **elevation of** approximately twice the level of ROS seen in the control samples, was observed after exposure to 15 μM MeHg. Greater MeHg concentrations did not lead to further increases in ROS levels (data not shown). $H_2O_2$ oxidizes DCFH in the presence of intracellular peroxidases (Myhre et al., 2003), and was used as a positive control. 100 μM $H_2O_2$ increased DCF fluorescence to 96 ± 4% above basal levels (Fig. 2, **insert**). There was no change in MeHg-induced increases in ROS levels when the experiment was repeated in calcium-free HEPES buffer containing 20 μM EGTA (Table 1).

### Effect of antioxidant treatment on MeHg-induced ROS formation and mitochondrial dysfunction

**The** synthetic antioxidant Trolox, a water-soluble analog of vitamin E, reduced the elevations in ROS following exposure to 5 μM MeHg from a 28.2% increase above control (without Trolox) to 20.4, 14.7, and 10.8%, at concentrations of 0.6, 3, and 6 μM, respectively (Fig. 3a). Based on these results, we chose 6 μM Trolox as the concentration for use in studies of Trolox modulation of MeHg-induced effects on mitochondrial metabolic function. **Because** Trolox concentrations of 0.6, 3, and 6 μM reduced background ROS levels by 12.2, 25.0, and 30.6%, respectively, all results were adjusted for the reduced background levels before statistical comparisons were made.

The lowest **concentration of** MeHg that significantly reduced mitochondrial metabolic function (5 μM MeHg) was chosen for investigation of the effect of Trolox in this assay. Incubation with 6 μM Trolox, a concentration that significantly decreased MeHg-induced ROS, did not affect MeHg-induced reductions in the MTT assay (Fig. **3b**).

### Effects of MeHg on cytosolic calcium levels

In normal HEPES buffer containing 1.2 mM $CaCl_2$, exposure to 1 or 5 μM MeHg significantly increased cytosolic calcium levels, compared to the vehicle control (ANOVA with repeated measures followed by Dunnett's test; $p \leq 0.001$) (Fig. 4). The increases in cytosolic calcium became apparent approximately 30 sec. after MeHg addition, and continued to rise throughout the duration of the experiment. This response was not altered by shifting to calcium-free HEPES buffer containing 20 μM EGTA before MeHg exposure (Table 1). The value of the 340 nm/380 nm fluorescence ratio at the start of the experiments was 1.25 ± 0.015 fluorescence units (mean of all samples ± SE at time = 0; n=15).

### Effects of MeHg on mitochondrial calcium levels

Exposure to 0.5, 1, or 5 μM MeHg significantly increased mitochondrial calcium **levels, compared** to the vehicle control (ANOVA with repeated measures followed by Dunnett's test; $\boldsymbol{p \leq 0.05}$**) (Fig. 5). Both unexposed** and vehicle controls maintained a stable rhod-2 fluorescence throughout 15 min, whereas in samples exposed to 0.5, 1.0, and 5.0 μM MeHg, fluorescence increased by 0.4, 0.6, and 1.1 fluorescence units, respectively. Rhod-2 fluorescence started at 2.84 ± 0.10 fluorescence units (mean ± SE of start value (time=0) for all samples, n=25).

## Discussion

### Effect of MeHg on mitochondrial function

In the present study we show that MeHg significantly alters mitochondrial function in striatal synaptosomes from adult rats, demonstrated by reductions in the conversion of MTT to formazan. This reaction is mainly catalyzed by succinate dehydrogenase in the mitochondria, although other dehydrogenases can also contribute. We have previously demonstrated a statistically significant correlation between mitochondrial function, assessed by MTT, and

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reductions in mitochondrial membrane potential (measured by JC-1) after MeHg exposure (Dreiem et al., 2005). Thus, the findings presented here suggest that, at MeHg concentrations ≥ 5 μM, mitochondrial function is impaired. These observations, obtained in striatal synaptosomes, are in agreement with results of previous studies **that used** cultured cerebellar granule cells, where exposure to 2.5 μM MeHg for 1 h reduced MTT metabolism to approximately 45% of control values (Castoldi et al., 2000).

## Effect of MeHg on ROS formation

In addition to changes in mitochondrial function, we also observed a significant increase in ROS production in striatal synaptosomes following MeHg exposure. The levels of ROS produced after exposure to high MeHg concentrations (7.5−15 μM) are **similar to those** seen after the addition of $H_2O_2$ which directly oxidizes DCFH. Indeed, in neuronal cultures, the addition of $H_2O_2$ at concentrations of 10−100 μM, causes cell death through oxidative damage (Ding and Keller, 2001, Gum et al., 2004, Ricart and Fiszman, 2001). Therefore, the high ROS levels observed after MeHg exposure in the present study further support the hypothesis that exposure to MeHg leads to toxicity in striatal synaptosomes. These findings are in agreement with results of several previous studies, using both *in vivo* (Ali et al., 1992, Yee and Choi, 1994) and *in vitro* systems, including cerebellar granule cells, cerebral cortical neurons, astrocytes, oligodendrocytes, and synaptosomes from whole brain (Bondy and McKee, 1990, Shanker and Aschner, 2003, Yee and Choi, 1996). Thus, the agreement between our present findings and these data, gathered in a variety of preparations, further validate the use of striatal synaptosomes in examining the biochemical sequelae following exposure to MeHg.

## Relationship between ROS formation and mitochondrial dysfunction

The fact that MeHg **reduced mitochondrial function and increased** ROS production suggests that the two processes **may be** causally linked. For example, ROS react rapidly with cellular macromolecules **and could cause damage to mitochondria**. **Indeed**, this sequence of events has been shown to occur in cultured cells through the addition of exogenous oxidants such as $H_2O_2$ (Ding and Keller, 2001). It is also possible, however, that MeHg-induced disruption of mitochondrial function could, instead, lead to ROS formation. To investigate this possibility, we measured mitochondrial function after MeHg exposure in the presence **or absence** of the antioxidant Trolox. If elevations in ROS reduce mitochondrial function, then scavenging MeHg-induced ROS by Trolox should either prevent or minimize these changes in mitochondrial function. Indeed Trolox significantly decreased ROS levels, but had no effect on MeHg-induced reductions in the MTT assay (Fig. 3**b**), **strongly suggesting that ROS are** not initiators of mitochondrial damage after exposure to MeHg. Rather, we suggest that the mitochondria themselves are the source of the ROS production. Indeed, superoxide anion radicals ($O_2^{\bullet -}$), formed by leakage of electrons from the mitochondrial electron transport chain to molecular oxygen, are major contributors to cellular ROS even during normal respiration; and $O_2^{\bullet -}$ production increases dramatically when mitochondria are damaged (Halliwell and Gutteridge, 1999). The present findings suggest that mitochondria are the primary target for MeHg and that ROS production could be a consequence of damage to the mitochondria rather than **being** an initiator of mitochondrial damage.

Previously, Trolox and other antioxidants have been shown to prevent several MeHg-induced effects, including ROS production, lipid peroxidation, changes in antioxidant enzyme activities, and cell death both *in vivo* and *in vitro* (Kasuya, 1975; Prasad et al., 1979; Bondy and McKee, 1990; Ususki et al., 2001; Belletti et al., 2002; Shanker and Aschner, 2003; Gatti et al., 2004). However, in other studies, antioxidants failed to prevent lipid peroxidation and cell death (Andersen and Andersen, 1993, Prasad et al., 1979, Sarafian and Verity, 1991). The discrepancies between these studies suggest that mechanisms, other than ROS formation, may be involved in MeHg toxicity. Indeed, our present results are in agreement with findings by

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Bondy and McKee (1990), who demonstrated that α-tocopherol offered no protection against MeHg-induced loss of mitochondrial membrane potential in rat whole-brain **synaptosomes (although they did not measure** ROS levels). Here, however, we clearly demonstrate that even though Trolox reduces MeHg-induced ROS to control levels, it provides no protection against MeHg-induced loss of mitochondrial function. Taken together, these findings indicate that ROS formation is a late event in MeHg toxicity, and that it is not the causative agent of the mitochondrial damage.

## Effect of MeHg on calcium homeostasis

Both mitochondrial dysfunction and ROS formation are closely linked with alterations in calcium homeostasis. For example, **although** mitochondria buffer cytosolic calcium levels via calcium uptake through the mitochondrial uniporter (Leo et al., 2005), a high mitochondrial calcium load can lead to mitochondrial damage and increased formation of mitochondrial ROS (Leo et al., 2005). We hypothesize that this sequence of events is responsible for the MeHg-induced reductions in mitochondrial function reported here. Indeed, we found not only that MeHg (1−5 μM) increased cytosolic calcium levels (Fig. 4), but that removal of extracellular calcium from the buffer did not prevent these increases. These results suggest that the MeHg-induced increases in cytosolic calcium are due to calcium release from intracellular stores. Our results are in partial agreement with data from previous studies in synaptosomes **where MeHg increased** the 340 nm/380 nm ratio of fura-2 in a time- and concentration-dependent manner (Denny et al, 1993). However, Denny and coworkers found a biphasic increase in the fura-2 emission ratio after MeHg exposure with a rapid first phase, which they attributed to elevations in non-calcium polyvalent cations, followed by a second gradual increase thought to be due to the influx of extracellular calcium. In our study no rapid first-phase increase in cytosolic calcium was observed and extracellular calcium did not contribute to the aforementioned increase in cytosolic calcium levels. These discrepancies may be due to the high MeHg concentrations (10−100 μM) used in the study by Denny and coworkers. Taken together, the findings of both groups suggest that differing mechanisms can operate depending on the MeHg concentration; intracellular calcium sources appear to be more important at lower concentrations, whereas influx of extracellular calcium may play a more important role at higher MeHg concentrations.

To further elucidate the role of calcium in MeHg-induced mitochondrial dysfunction, we used the fluorescent indicator rhod-2 to measure mitochondrial calcium levels after MeHg exposure. MeHg increased mitochondrial calcium levels over approximately the same molar range (0.5 −5 μM) in which it increased cytosolic calcium levels. Elevated mitochondrial calcium can disrupt the mitochondrial membrane potential and is associated with opening of the mitochondrial permeability pore, an event that inexorably leads to loss of mitochondrial function and cell death (Stavrovskaya and Kristal, 2005). MeHg-induced opening of the mitochondrial permeability pore has been indirectly demonstrated via blockade of the effects of MeHg with cyclosporin A (Bragadin et al., 2002, Limke and Atchison, 2002, Limke et al., 2003).

The elevations in mitochondrial calcium levels we report here are in agreement with **those** of Limke and coworkers (2003), who demonstrated that exposure of cultured cerebellar granule cells to 0.5 μM MeHg increased mitochondrial calcium levels. **Our findings strongly suggest** that released mitochondrial calcium is most likely not the source of the increased cytosolic calcium levels: **if this was the case,** we would predict a reduction, rather than an elevation, in mitochondrial calcium levels. Instead, this finding suggests that elevations in cytosolic calcium may be due to releases from other intracellular sources, such as synaptic vesicles, smooth endoplasmatic reticulum (SER), and calcium-binding proteins that, in turn, contribute to the elevations in mitochondrial calcium.

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To investigate the role of **extracellular** calcium in MeHg-induced mitochondrial dysfunction, we measured ROS levels and mitochondrial function in MeHg-exposed striatal synaptosomes **in calcium-free buffer with EGTA**. Neither MeHg-induced mitochondrial dysfunction nor ROS formation were affected by manipulation of **extracellular calcium levels** (Table 1). Previous studies have shown that cytosolic calcium levels increase after MeHg exposure, and that **preventing** these calcium increases can either attenuate or delay cell toxicity (Gasso et al., 2001,Limke et al., 2003). The findings of the present study suggest that extracellular calcium is not necessary for MeHg-induced toxicity in striatal synaptosomes, but rather suggest that intracellular calcium, and especially increased mitochondrial calcium levels, play a role in MeHg-induced mitochondrial damage.

In conclusion, we have shown that MeHg-induced mitochondrial dysfunction is not a result of increased ROS levels; rather, we suggest that ROS production is a secondary event in MeHg toxicity, **and a consequence of** MeHg-induced mitochondrial damage. Furthermore, we observed a correlation between mitochondrial dysfunction and increased mitochondrial calcium levels, suggesting that MeHg-induced elevations in mitochondrial calcium are responsible for the observed mitochondrial damage following MeHg exposure.

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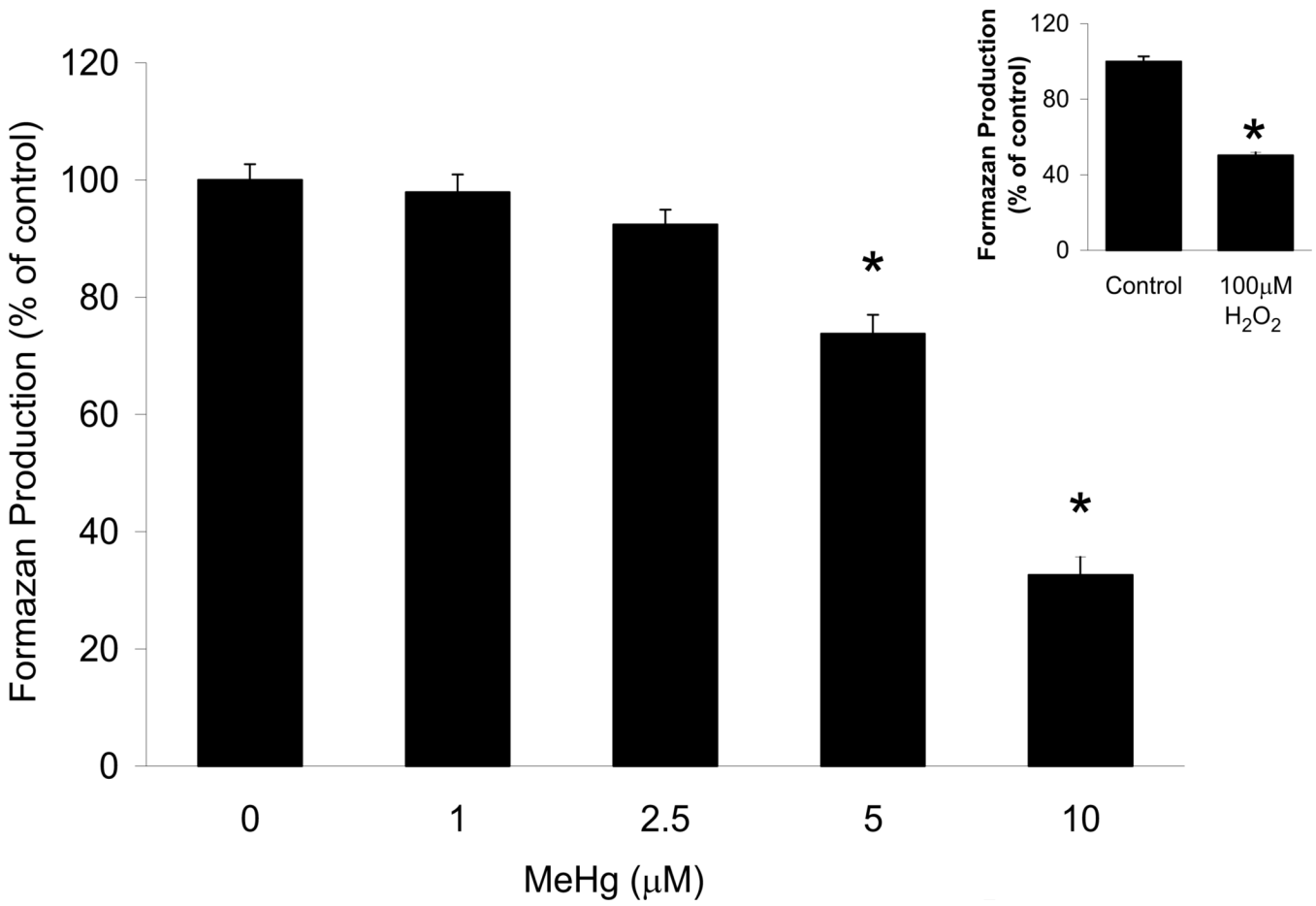


**Fig. 1.**

MeHg decreases mitochondrial metabolic function in rat striatal synaptosomes. Synaptosomes were exposed to MeHg for 30 min, and mitochondrial function was then assessed by measurement of the conversion of MTT to formazan. The results are given as percentage of vehicle controls (set to 100%). The values are mean of eight or nine wells in three independent experiments ± SE. * denotes significantly different from control (ANOVA, Dunnett's test; $p \leq 0.05$). The insert shows the effect of 100 μM $H_2O_2$, which was used as a positive control.

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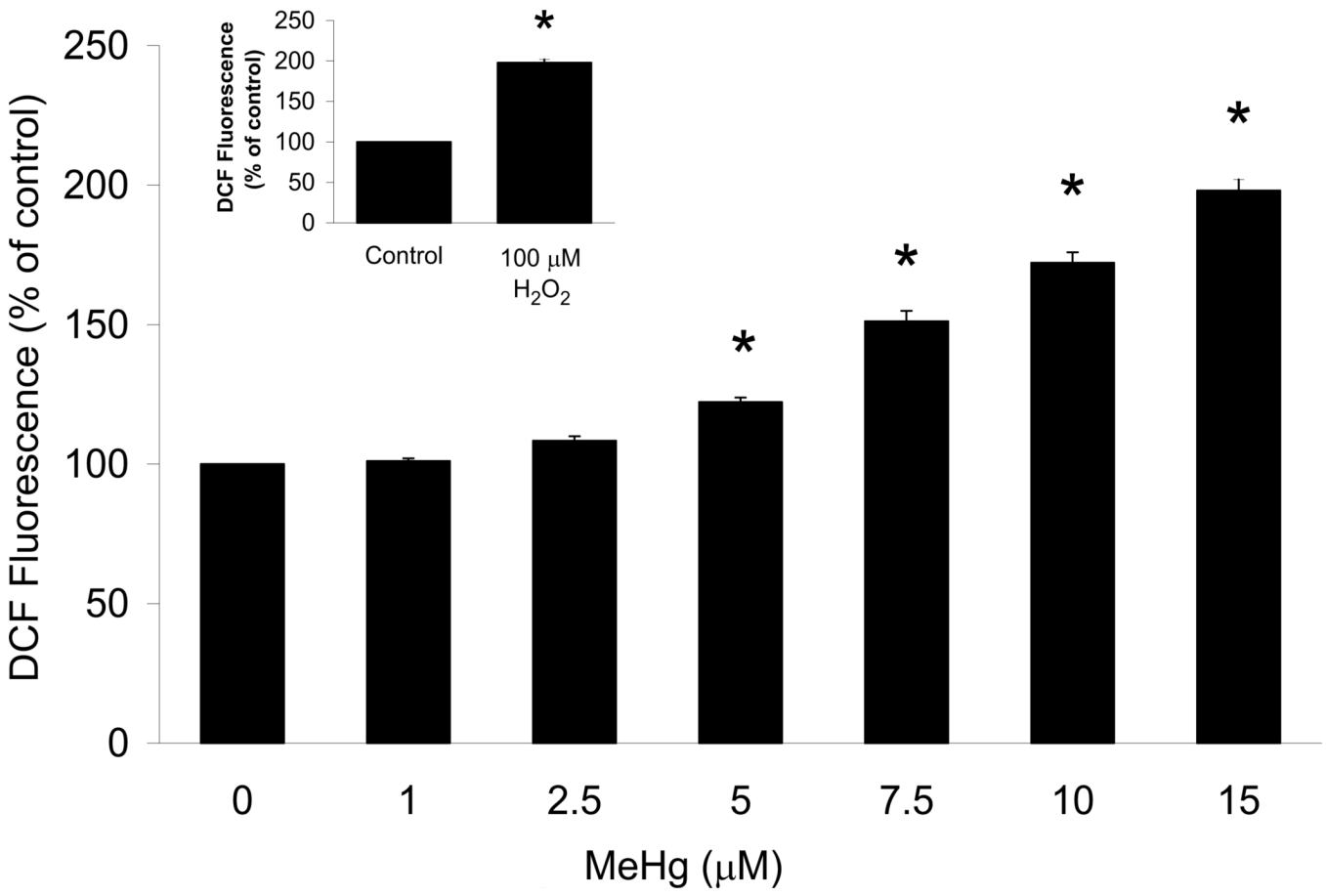


**Fig. 2.**

MeHg increases ROS formation in rat striatal synaptosomes. Striatal synaptosomes were loaded with DCFH-DA and exposed to MeHg for 30 min, before ROS formation was assessed by measurement of the increase in DCF fluorescence. The results are given as percentage of vehicle controls (set to 100%). The values are mean of 16 to 22 wells in three to four independent experiments ± SE. * denotes significantly different from controls (ANOVA, Dunnett's test; $p \leq 0.05$). The insert shows the effect of 100 μM $H_2O_2$, which was used as a positive control.

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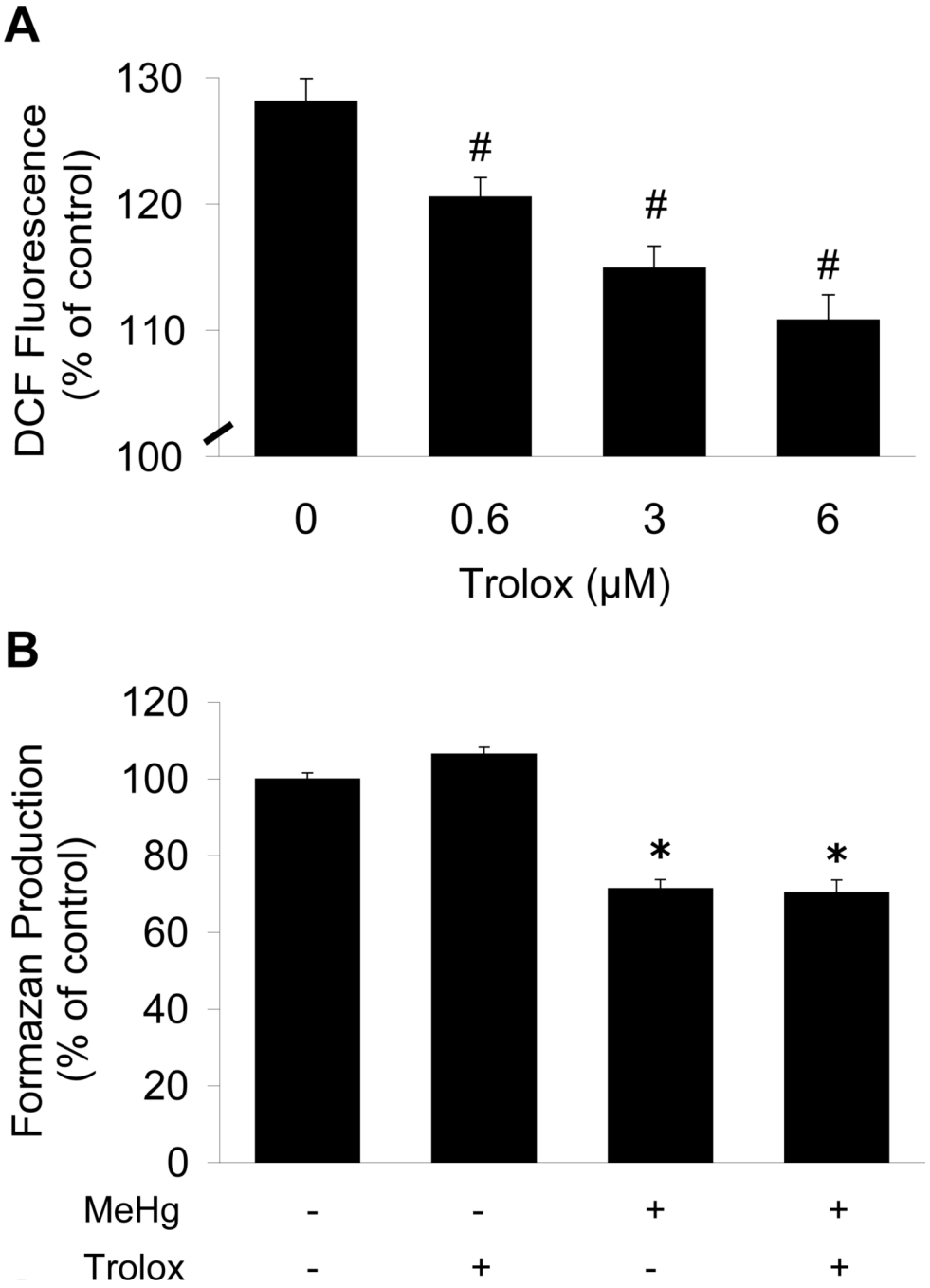


**Fig. 3.**

Trolox reduces MeHg-induced ROS but does not affect MeHg-induced loss of mitochondrial function. a) Rat striatal synaptosomes were exposed to 5 μM MeHg in the presence of various concentrations of Trolox; ROS levels were then assessed by measurement of the increase in DCF fluorescence. b) Rat striatal synaptosomes were exposed to 5 μM MeHg in the presence of 6 μM Trolox, and mitochondrial function was assessed by measurement of formazan production (MTT assay). The results are given as percentage of the appropriate control (set to 100 %). *denotes significantly different from control, and # denotes significantly different from 5 μM MeHg without Trolox (ANOVA, Dunnett's test; $p \leq 0.05$).

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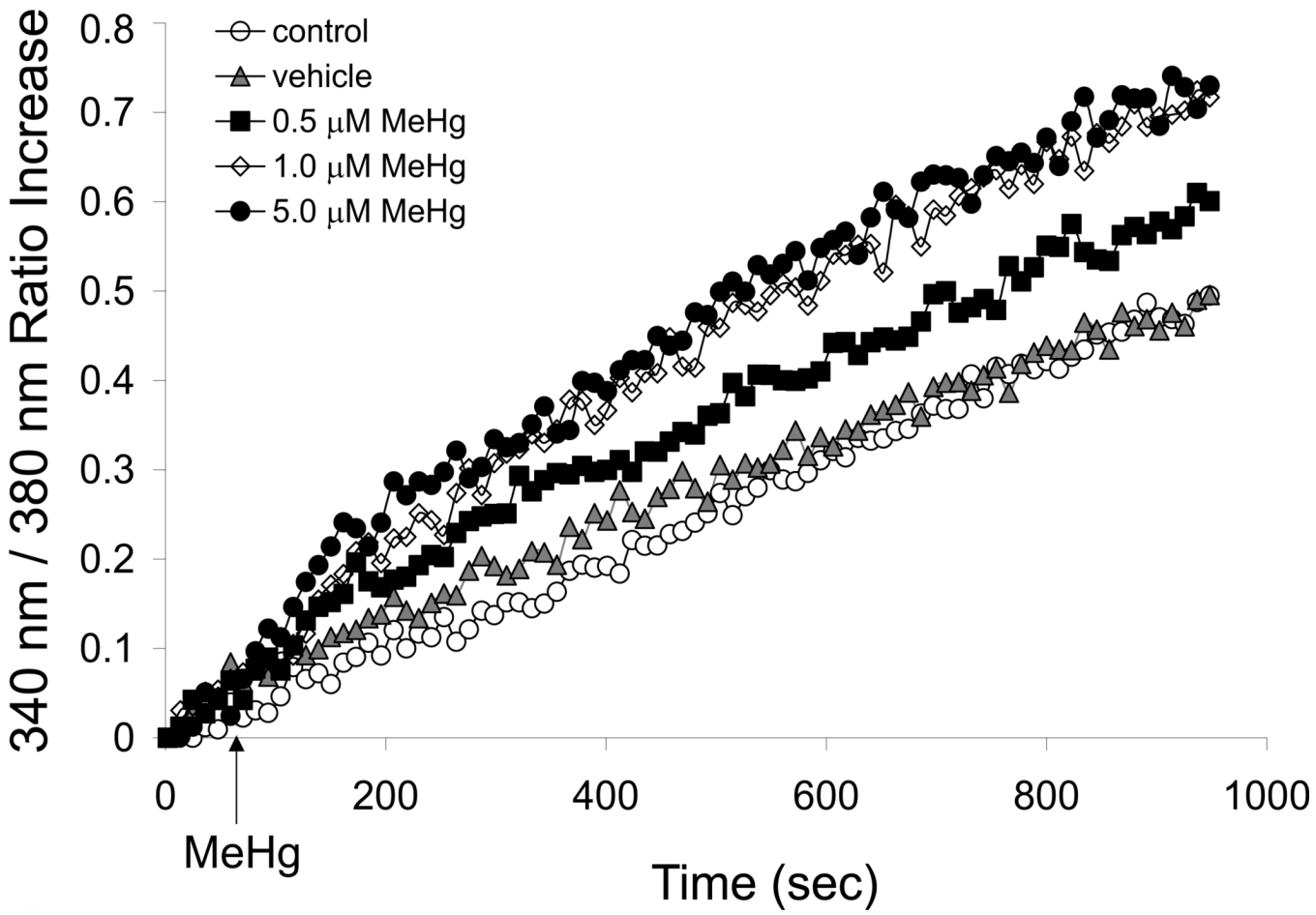


**Fig. 4.**

MeHg exposure increases cytosolic calcium levels in rat striatal synaptosomes. MeHg was added to synaptosomes (arrow) preloaded with Fura-2, and the levels of cytosolic calcium were assessed by monitoring of the 340 nm/380 nm fluorescence ratio of fura-2. The figure shows the means of three independent experiments after subtraction of starting levels.

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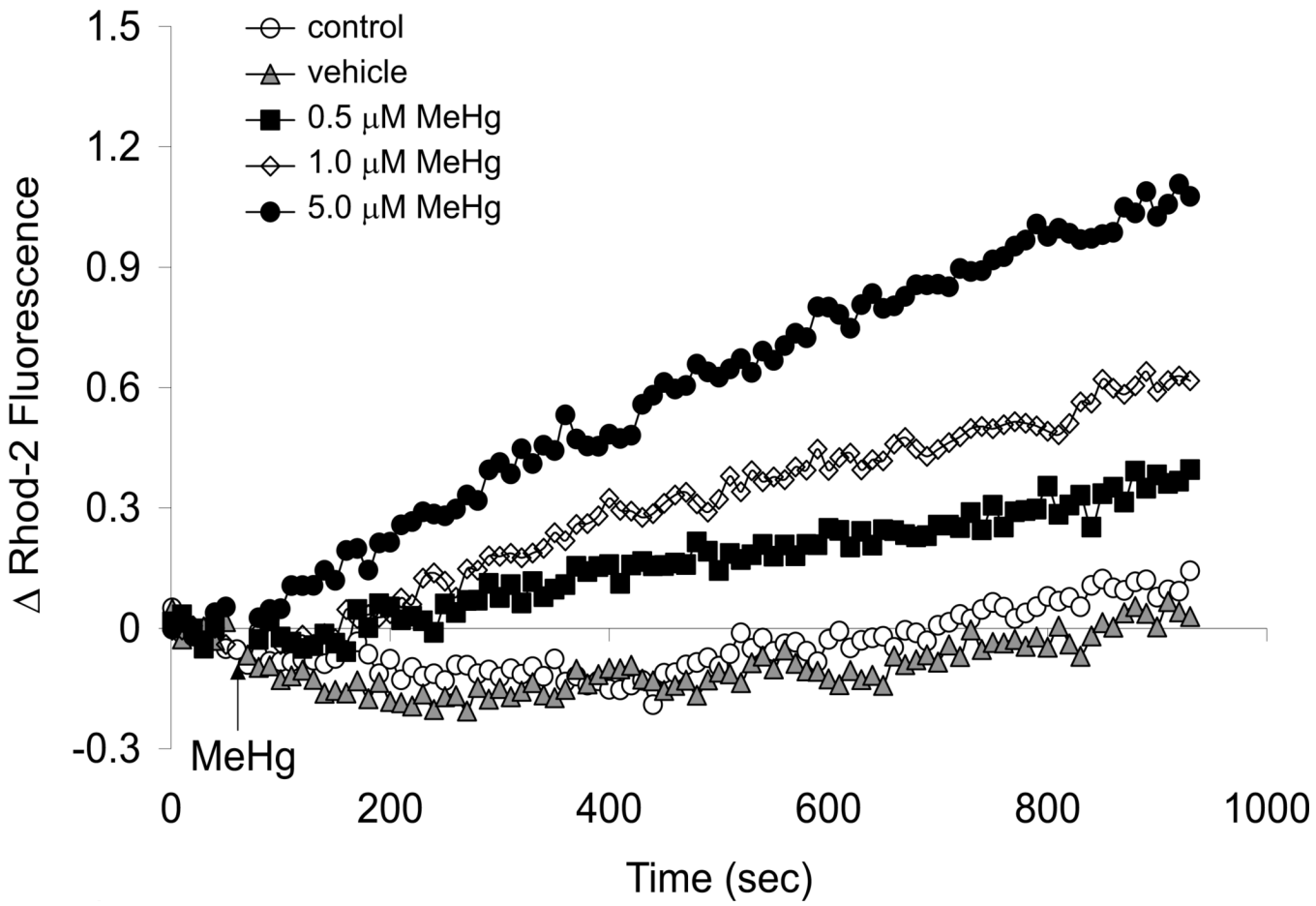


**Fig. 5.**

MeHg exposure increases mitochondrial calcium levels in rat striatal synaptosomes. MeHg was added to synaptosomes (arrow) preloaded with rhod-2, and the levels of mitochondrial calcium were assessed by monitoring of the 580 nm fluorescence of rhod-2. The figure shows the means of five independent experiments after subtraction of starting levels.

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**Table 1**

Effect of removal of extracellular calcium on MeHg-induced ROS Formation (DCF fluorescence), mitochondrial function (MTT assay) and cytosolic calcium (fura-2 fluorescence).

| | DCF fluorescence (% of control) | MTT assay (% of control) | Fura-2 fluorescence* (340/380 ratio increase) |
|---|---|---|---|
| HEPES | 122.4 +/− 2.5 | 34.0 +/− 3.4 | 0.52 +/− 0.04 |
| $Ca^{2+}$-free HEPES w. 20 μM EGTA | 127.3 +/− 2.1 | 35.3 +/− 3.0 | 0.54 +/− 0.02 |

Note: All values are mean +/− SE of 3 independent experiments.

*Ratio increase 15 min after MeHg addition.