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Triethylene glycol dimethacrylate impairs bioenergetic functions and induces oxidative stress in mitochondria *via* inhibiting respiratory Complex I

Krisztina Mikulás[a,b], *Péter Hermann*[a], *István Gera*[c], *Timea Komlódi*[b], *Gergő Horváth*[b], *Attila Ambrus*[b], *László Tretter*[b,*]

[a] *Department of Prosthodontics, Semmelweis University, Budapest, Hungary*
[b] *Department of Medical Biochemistry, MTA-SE Laboratory for Neurobiochemistry, Semmelweis University, Budapest, Hungary*
[c] *Department of Periodontology, Semmelweis University, Budapest, Hungary*

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ABSTRACT

Objectives. Earlier studies demonstrated that dental resin monomers lower cellular viability and provoke oxidative stress. Reactive oxygen species (ROS) formation has a key role in triethylene glycol dimethacrylate (TEGDMA) induced adverse reactions. In the present study the effects of TEGDMA on mitochondrial functions were investigated to identify a direct molecular target for cytotoxicity.

Methods. Mitochondria were isolated from guinea pig brain. The most important bioenergetic parameters, oxygen consumption, membrane potential ($\Delta\Psi_m$), and ATP production were assessed. Mitochondrial H_2O_2 production and elimination and the NAD(P)H level reported on redox balance.

Results. Mitochondria were supported with respiratory substrates to be oxidized by either Complex I (CI) or Complex II (CII). $\Delta\Psi_m$ was depolarized, respiration and ATP production was greatly diminished when applying CI substrates in the presence of TEGDMA. The same parameters remained essentially unaffected when CII substrate *plus* TEGDMA were applied. H_2O_2 production by mitochondria was significantly stimulated by TEGDMA in the presence of CI substrates. In the presence of TEGDMA mitochondrial elimination of exogenous H_2O_2 was impaired. When CII substrate supported the mitochondria in the absence of ADP the H_2O_2 generation was decreased. NADH autofluorescence results also demonstrated the inhibitory effect of TEGDMA on CI activity.

Abbreviations: AK, adenylate kinase; ANT, adenine-nucleotide translocator; α-KG, alpha-ketoglutarate; α-KGDH, alpha-ketoglutarate dehydrogenase; AP5, P^1,P^5-di(adenosine-5′) pentaphosphate; BSA, bovine serum albumin; CAT, carboxyatractylate; $\Delta\Psi_m$, mitochondrial membrane potential; DMSO, dimethyl-sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FCCP, carbonylcyanide-*p*-trifluoromethoxy-phenylhydrazone; GM, glutamate *plus* malate; OXPHOS, oxidative phosphorylation; SLP, substrate-level phosphorylation; Succ, succinate.

* *Corresponding author at*: Department of Medical Biochemistry, Semmelweis University, Tűzoltó st. 37-47, H-1094 Budapest, Hungary.
E-mail address: tretter.laszlo@med.semmelweis-univ.hu (L. Tretter).

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Significance. TEGDMA inhibits CI in the respiratory chain, which explains effects induced by TEGDMA on redox homeostasis, apoptotic and necrotic cell deaths described in previous studies. Identification of the molecular target of TEGDMA may influence the development of relevant biomaterials and may induce new therapeutic strategies to control the adverse effects of resin monomers.

1. Introduction

Dental composite resins are commonly used to restore structural tooth damages. The development of resin-based composites (RBCs) has resulted in esthetically pleasing and long lasting dental restorations [1]. Recently, biocompatibility of the filling materials has become a clinically relevant issue [2]. Most RBCs contain strongly viscous major monomers and co-monomers of lower viscosity such as triethylene glycol dimethacrylate (TEGDMA) [3–5]. Incomplete RBC polymerization may lead to an undesired leaching of the monomers into the oral tissues [6,7]. TEGDMA is of amphiphilic character and the most common co-monomer released from polymerized composites [5,8–10]. Shortly after the polymerization process was considered to be completed, both 2-hydroxyethyl methacrylate (HEMA) and TEGDMA were detectable in the saliva in millimolar concentrations [5,6,11,12]. After months and years, mechanical deterioration and hydrolytic disintegration by unspecific salivary esterases may induce further release of the resin monomers, which are able to cross the dentin, enter the pulp, and reach concentrations that may indeed affect cellular functions [8,13]; the concentration of TEGDMA in the pulp was shown to reach levels of up to 4 mM [11,14,15]. The systemic blood concentrations of the monomers are orders of magnitude lower [6], therefore the probability of the development of TEGDMA-induced adverse systemic effects is negligible [9]. TEGDMA and HEMA were reported to affect various cellular functions and the viability of the cells in the oral cavity [12,16–18]. It was reported earlier that TEGDMA could initiate cell stress responses, induce reactive oxygen species (ROS) generation, and cause glutathione depletion in a wide variety of eukaryotic cells *in vitro* [12,19,20]. The genotoxic [21] and mutagenic effects of TEGDMA are likely consequences of ROS-triggered DNA damages; the toxic effects could apparently be eliminated applying ROS scavengers [18,22–25]. It was demonstrated that TEGDMA could induce, depending on the concentration, apoptotic [26,27] or necrotic cell death [27–30].

These results in the literature raise the possibility of a common mechanism and perhaps molecular target which would be responsible for the observed cellular changes, however, the underlying mechanism behind these phenomena remains poorly understood. Recently the involvement of NADPH oxidase 4 (NOX 4) in TEGDMA-induced apoptosis and ROS generation was described [31]. Earlier a collapsed mitochondrial membrane potential and increased ROS formation were detected in gingival fibroblasts after a treatment with TEGDMA [32]. Mitochondrial dysfunction certainly influences cell viability as the decreased ATP level can lead to necrotic cell death. Mitochondrial impairment can increase the level of ROS, provoke DNA damages, and consequently induce apoptosis [33]. Therefore, it appeared to be plausible to seek after mitochondrial targets in order to understand the mechanism of TEGDMA-induced toxicity. Taking into account that mitochondrial impairment can either be primary or secondary, and that the distinction is not always clear in a cellular system, isolated mitochondria were chosen as the objects of this study. According to our hypothesis, most of the toxic effects of TEGDMA could potentially be explained by the development of bioenergetic insufficiency and increased ROS generation. The hypothesis that mitochondrial dysfunctions could be responsible for the onset of either necrotic [34,35] or apoptotic cell death [36] does not demand any sophisticated verification. Low doses of Complex I (CI) inhibitors generally induce apoptosis, while higher doses of the same compound may evoke necrotic cell death [37].

The role of mitochondria in cellular generation of ROS is also well known [38]. Various mechanisms and molecular entities can be responsible for the enhanced ROS generation of mitochondria [39,40].

In the present study, low millimolar concentrations of TEGDMA were used to challenge isolated brain mitochondria and the most important bioenergetic functions were evaluated in parallel with the measurement of mitochondrial H_2O_2 homeostasis. We propose that inhibition of the mitochondrial respiratory CI may be responsible for most of the cytotoxic effects of TEGDMA.

2. Materials and methods

2.1. *Preparation of brain mitochondria*

Animal experiments were performed in accordance with the relevant guidelines of the National Institute of Health (USA) and the Semmelweis University. Mitochondria were isolated from guinea pig brain cortex using a Percoll gradient as detailed elsewhere [41]. Unless otherwise indicated, 0.1 mg/mL mitochondrial protein concentration was applied for all the experiments.

2.2. *Incubation medium*

Measurements were carried out at 37 °C in an assay medium containing the following components: 125 mM KCl, 20 mM HEPES, 2 mM K_2HPO_4, 1 mM $MgCl_2$, and 0.025 (w/v) % bovine serum albumin (BSA; free of fatty acids). Mitochondria were energized by either alpha-ketoglutarate (α-KG), or glutamate

plus malate (GM), or succinate (Succ), all in 5 mM concentration.

2.3. Measurement of mitochondrial oxygen consumption

Mitochondrial respiration was monitored using the high-resolution respirometry system Oxygraph-2k [42] (Oroboros Instruments, Innsbruck, Austria). Oxygen sensors were calibrated at air-saturation and in oxygen-depleted media.

2.4. Measurement of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial $\Delta\psi_m$ was detected using safranine O (2 μM). This lipophilic cationic dye gets distributed among mitochondria and the surrounding medium; the value of the partition coefficient is the function of the actual mitochondrial $\Delta\psi_m$. Measurements were performed using a Hitachi F-4500 spectrophotometer (Hitachi High Technologies, Maidenhead, UK) at 495 and 585 nm excitation and emission wavelengths, respectively, as described previously [43].

2.5. Parallel measurements for H_2O_2 and NAD(P)H

H_2O_2 formation by isolated mitochondria was detected using the Amplex Ultra Red fluorescence assay (for review, see Ref. [44]). In this assay, Amplex Ultra Red (3 μM) is converted to the highly fluorescent compound resorufin by H_2O_2 and horseradish peroxidase (5 U/2 mL). Fluorescence was detected using a PTI Deltascan fluorescence spectrophotometer (Photon Technology International, Lawrenceville, NJ, USA); for excitation and emission, the 550 and 585 nm wavelengths were applied, respectively. Each measurement was calibrated by 100 pmol H_2O_2 at the end of the experiment. In a parallel experiment, using the double-excitation/double-emission mode of the spectrofluorimeter, NAD(P)H fluorescence was also measured at 340 and 466 nm excitation and emission wavelengths, respectively.

2.6. Measurement of mitochondrial H_2O_2 elimination

Mitochondrial elimination of H_2O_2 was measured as previously described [45]. Briefly: glutamate *plus* malate supported mitochondria (0.1 mg/mL) were preincubated in either the presence or absence of TEGDMA (5 mM) or DMSO for 5 min. At 5 min H_2O_2 (10 μM) was given, then 50 μL samples were taken at 30 s intervals for 2 min, and the residual H_2O_2 was determined by Amplex Ultra Red fluorimetry, as described in the previous Section 2.5. Measurements were calibrated with known amounts of H_2O_2.

2.7. Measurement of reduced glutathione consumption

The measurement of reduced glutathione (GSH) is based on the reaction of the thiol SH group with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Freshly diluted GSH (2 mM) was incubated in the reaction medium at 37 °C for various time intervals. Aliquots were taken and the generated TNB was measured *via* spectrofotometry at 412 nm [46].

2.8. Measurement of ATP production

ATP production by isolated mitochondria was detected using a coupled enzyme assay that applies hexokinase and glucose-6-phosphate dehydrogenase, as described previously [47]. The assay medium was supplemented with 3 mM $NADP^+$, 1.5 U hexokinase, 0.5 U glucose-6-P dehydrogenase, 5 mM glucose, and 200 μM 2-amino-phosphono-phentanoate (an adenylate kinase inhibitor). Mitochondria (0.05 mg/mL), ADP (2 mM), glutamate *plus* malate (5–5 mM) or succinate (5 mM), and TEGDMA (5 mM) were added as indicated in the corresponding figures. ADP was converted to ATP, which left the mitochondrion *via* the adenine nucleotide translocase (ANT). In the medium, ATP phosphorylated glucose *via* hexokinase and the produced glucose-6-phosphate got further converted to 6-phosphogluconate *via* glucose-6-phosphate dehydrogenase; with the concomitant reduction of $NADP^+$ to NADPH. Light absorbance of NADPH was recorded at 37 °C and 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$) using a JASCO spectrophotometer (ABL&E-JASCO V-650, Tokyo, Japan).

2.9. Detection of mitochondrial respiratory Complex I activity

For the Complex I assay the method described by Ragan and Hatefi [48] was used. The assay measures the rate of the conversion of NADH to NAD^+ while the electron acceptor is Coenzyme Q1 (CoQ1). Mitochondria were incubated in the standard medium and subsequently frozen and thawed three times. Thereafter, 200 μg was transferred into the following medium: 100 mM KH_2PO_4, 40 μM Coenzyme Q_1, 1 mM KCN, pH 7.4. 50 μM NADH initiated the reaction, which was repeated in the presence of either 5 mM TEDGMA or 1 μM rotenone. The initial rate of the change in absorbance at 340 nm was detected at 37 °C using a JASCO spectrophotometer.

2.10. Chemicals

TEGDMA was obtained from VOCO GmbH (Cuxhaven, Germany) and dissolved in dimethyl sulfoxide (DMSO); the 1 M stock solution was stored at −20 °C. The purity of triethylene glycol dimethacrylate was 97%; the remaining 3% was triethylene glycol monomethacrylate. The content of UV inhibitor is 150 ppm HQME (hydroquinone monomethyl ether) (0,015%), which is usual for a methacrylate monomer.

Standard laboratory chemicals were purchased from Sigma (St. Louis, MO, USA). The Amplex Ultra Red reagent was from Molecular Probes (Eugene, OR, USA).

2.11. Statistics

Pairwise comparisons were evaluated by Student's t-test. Statistical differences for multiple comparisons were evaluated using the one-way ANOVA method, or for data not following normal distribution ANOVA on ranks Kruskar Wallis test was applied. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. The effect of TEGDMA on mitochondrial respiration

Mitochondria were energized with by either alpha-ketoglutarate (α-KG), or glutamate *plus* malate (GM) or succinate (Succ) as respiratory substrate. NADH is formed when either α-KG or GM gets oxidized inside the mitochondrion. Since the produced NADH is oxidized by CI, α-KG and GM are both considered to be Complex I substrates; whereas oxidation of succinate is predominantly associated with Complex II. Oxidative phosphorylation (OXPHOS) was initiated by ADP (2 mM). Afterwards, mitochondria were challenged with TEGDMA. Experiments were terminated with carboxyatractylate (CAT) (2 μM) which inhibits ADP-ATP exchange [49].

3.1.1. Oxidation of Complex I substrates

In the presence of ADP oxygen consumption was increased from 12 ± 1 to 98 ± 2 nmol/min/mg protein in α-KG-supported mitochondria (Fig. 1A for original traces and Fig. 1B). The high respiratory control ratio (RCR: oxidation rate in the presence of ADP *vs* oxidation rate in the absence of ADP) indicated the high quality of mitochondrial preparations used in this study (RCR > 7). A subsequent administration of TEGDMA (5 mM) resulted in 69% decrease in oxygen consumption (from 98 ± 2 to 30 ± 2 nmol/min/mg protein) (Fig. 1A,B). Challenging mitochondria with an ANT inhibitor decreased the α-KG-supported respiration both in the absence and presence of TEGDMA (Fig. 1A,B). In GM-energised mitochondria, respiration was stimulated by ADP from 25 ± 2 to 200 ± 9 nmol/min/mg protein. Similarly to the effect observed with α-KG, addition of TEGDMA (5 mM) decreased the rate of the ADP-stimulated respiration by 88% (from 200 ± 9 to 24 ± 3 nmol/min/mg protein). Significant effect of TEGDMA on ADP-stimulated O_2 consumption was detected from 2 mM concentration (Fig. 1C). CAT lowered the rate of oxygen consumption both in TEGDMA-treated and non-treated mitochondria.

3.1.2. Oxidation supported by a Complex II substrate

Unlike with the NADH-linked substrates, in succinate-supported mitochondria TEGDMA given after ADP did not influence significantly the rate of oxygen consumption (from 166 ± 10 nmol/min/mg protein to 159 ± 5 nmol/min/mg protein) (Fig. 1D). These results unequivocally imply that TEGDMA does not inhibit Complex II function.

3.2. Effect of TEGDMA on the mitochondrial membrane potential ($\Delta\psi_m$)

The $\Delta\psi_m$ is an important parameter of mitochondrial bioenergetic competence, whose building up is a prerequisite for efficient OXPHOS-dependent ATP synthesis. $\Delta\psi_m$ was measured in isolated mitochondria supported by either α-KG or GM or Succ. The experimental protocol was identical to that of the oxygen consumption measurement (Section 3.1). In the presence of respiratory substrates, the inner membrane became hyperpolarized (Fig. 2), indicated by a decrease of safranin fluorescence. $\Delta\psi_m$ was higher in GM-supported mitochondria than in mitochondria respiring on α-KG, reflecting the higher rate of oxidation of GM (Figs. 2A,B and 1B,C). Subsequent addition of ADP caused depolarization with both substrates. Both in α-KG and in GM supported mitochondria, TEGDMA induced further depolarization (Fig. 2A,B *trace b*). Addition of CAT to TEGDMA-treated mitochondria resulted in a complete depolarization, as opposed to control mitochondria where CAT induced membrane hyperpolarization. The total depolarization of mitochondria was achieved using uncoupler carbonylcyanide-*p*-trifluoromethoxy-phenylhydrazone (FCCP) (250 nM). Contrary to that observed with CI substrates in succinate supported mitochondria TEGDMA did not depolarize mitochondria in the presence of ADP and subsequent addition of CAT resulted in an elevation of $\Delta\psi_m$ both in TEGDMA-treated mitochondria and in control condition (Fig. 2C). Consequently $\Delta\Psi_m$ measurements also support the notion that Complex I activity is selectively inhibited by TEGDMA.

3.3. The effect of TEGDMA on H_2O_2 generation and elimination

The prominent role of mitochondria in modulation of redox homeostasis is widely accepted [40,50]. Mitochondria participate not only in ROS generation, but also in ROS detoxication (for review see Ref. [44]). In the following experiments, mitochondrial H_2O_2 production and elimination were both measured in the presence of CI substrates and H_2O_2 production was also measured with non-CI substrates like Succ and alpha-glycerophosphate (α-GP).

3.3.1. H_2O_2 formation in mitochondria supported by NADH-linked substrates

GM and α-KG both initiated H_2O_2 production in mitochondria, as demonstrated in Fig. 3A,B. Addition of TEGDMA elevated ROS production by 17% (from 358 ± 16 to 420 ± 25 pmol/min/mg protein) (Fig. 3B). Addition of ADP resulted in a reduction in the rate of H_2O_2 production [41,51] in mitochondria energized by either α-KG or GM, due to depolarization of the inner mitochondrial membrane. TEGDMA augmented the rate of H_2O_2 production by 18% (from 81 ± 2 to 99 ± 4 pmol/min/mg protein) in α-KG-supported mitochondria (Fig. 3A), and by 74% (from 84 ± 6 to 146 ± 8 pmol/min/mg protein) in GM-supported mitochondria (Fig. 3B and B inset). Significant stimulation of H_2O_2 production has already been observed at 2 mM TEGDMA (Fig. 3B). The addition of CAT caused remarkable stimulation of H_2O_2 formation both in the presence and absence of TEGDMA. It is known that the horseradish peroxidase (HRP)-Amplex Ultra Red system not only detects H_2O_2 formation, but it also eliminates H_2O_2 [44] mitigating the H_2O_2-mediated oxidative stress. In order to assess the effect of TEGMA without this interference, mitochondria were pretreated with TEGDMA for 10 min in the presence of glutamate *plus* malate and the absence of Amplex *plus* HRP.

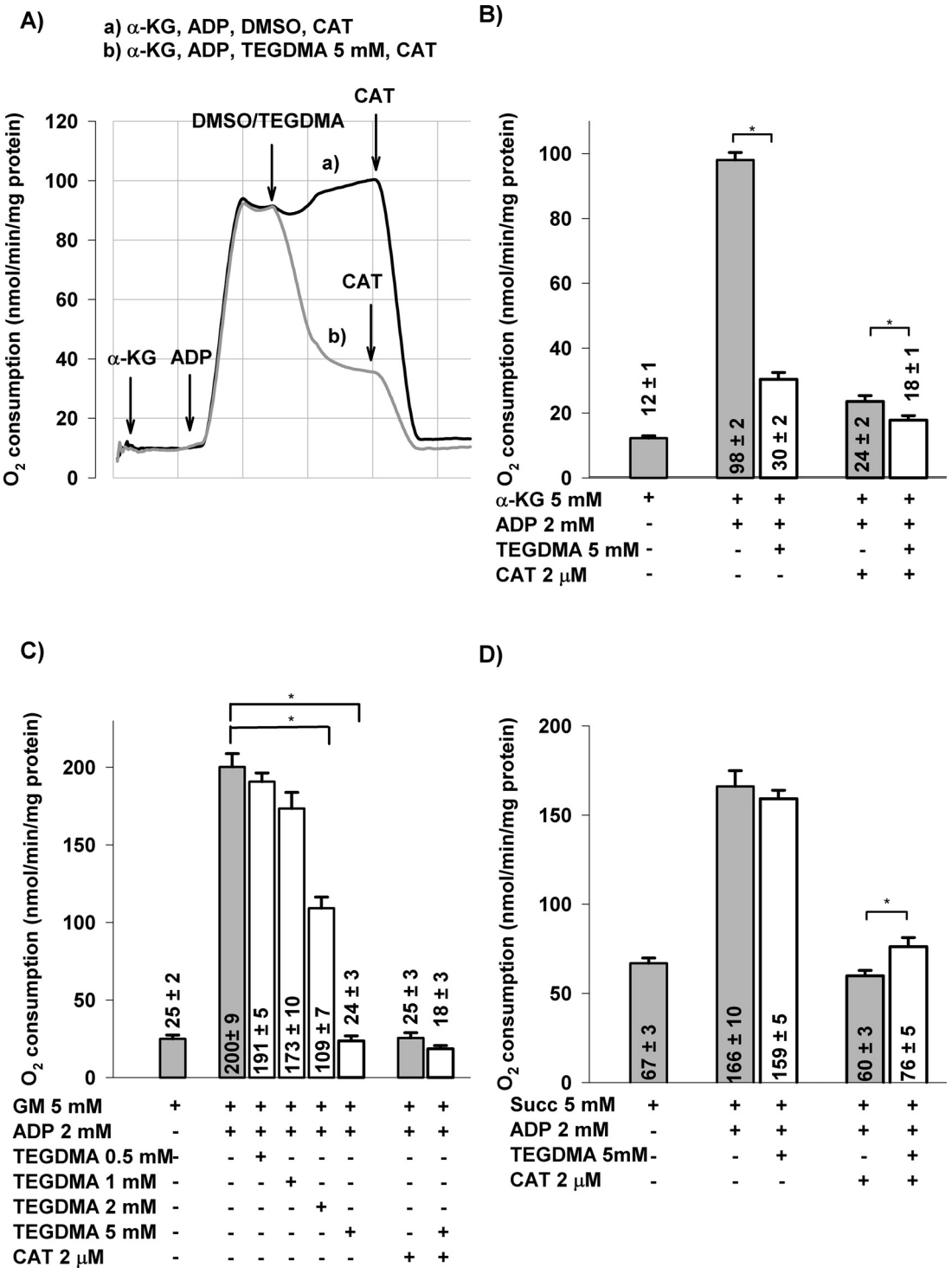

Fig. 1 – Effects of TEGDMA on the O_2 consumption rate of the isolated brain mitochondria in the presence of various respiratory substrates: α-ketoglutarate (α-KG; A,B), glutamate *plus* malate (GM; C), or succinate (Succ; D). Mitochondria were incubated in the 'standard medium' described under Section 2. ADP, TEGDMA (*gray curve*), DMSO (*black curve*), and carboxyatractylate (CAT) were given as indicated on the original traces (1A). The effects of TEGDMA (*white bars*) (*with various concentrations in GM-supported mitochondria as well 1C*) are compared to controls (*gray bars*, no TEGDMA only DMSO was added). Oxygen consumption, written on the bars, is expressed in nmol/min/mg protein (mean ± SEM). Significant differences are indicated $^*p < 0.05$.

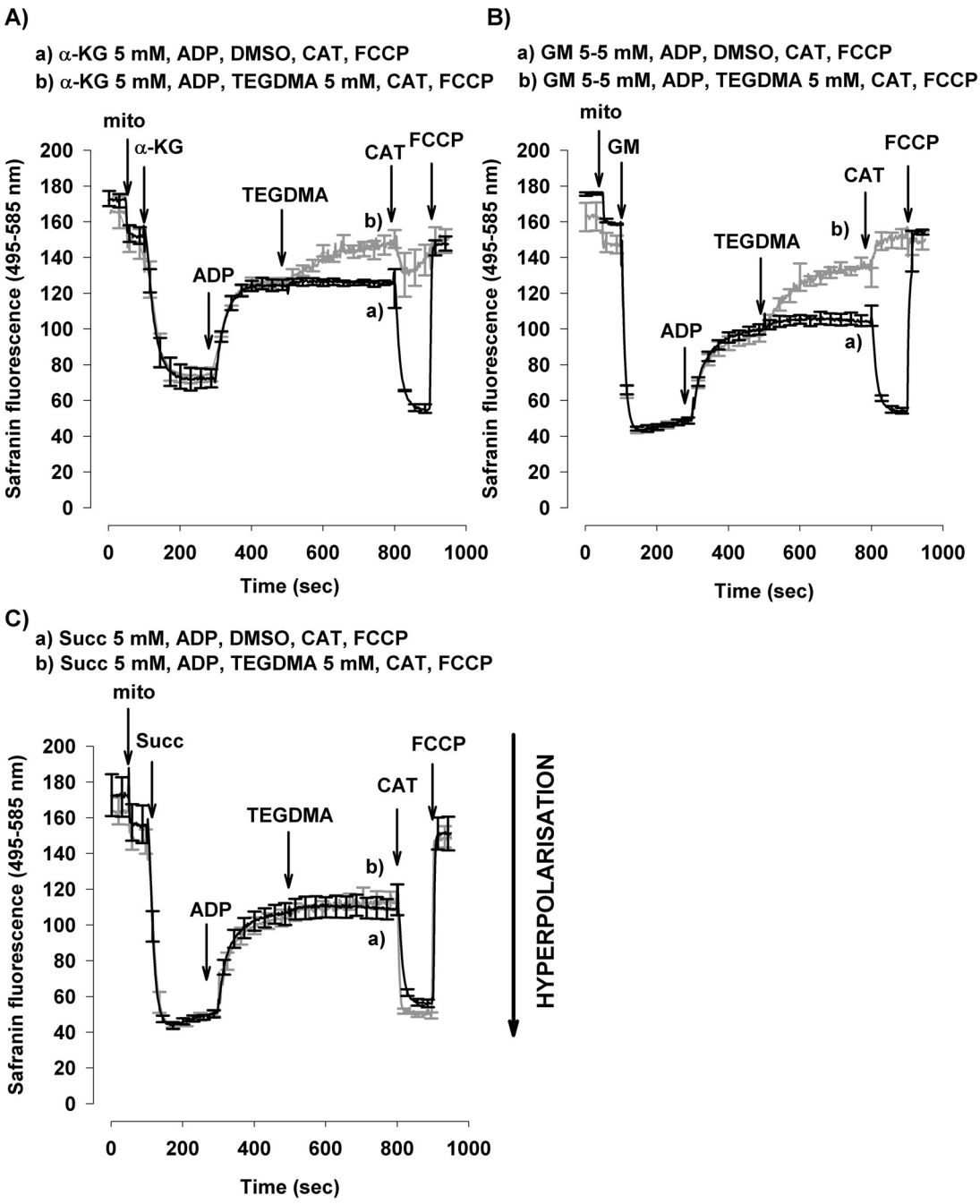

Fig. 2 – The effect of TEGDMA on Δ Ψ_m in α-ketoglutarate (A), glutamate plus malate (B) and succinate (C) respiring brain mitochondria. Mitochondria (0.1 mg/mL) were incubated in standard medium as described in Section 2. TEGDMA (5 mM), α-ketoglutarate (α-KG; 5 mM), glutamate plus malate (GM; 5–5 mM), succinate (Succ; 5 mM), ADP (2 mM), carboxyatractylate (CAT; 2 μM) and FCCP (250 nM) were added as indicated. The effect of TEGDMA (*dark grey; trace b*) is compared with controls (without TEGDMA black; *trace a*). Traces represent the average ± SEM of four independent experiments.

3.3.2. *Effect of pretreatment with TEGDMA on ROS production*

After the 10 min preincubation, H_2O_2 production was measured by Amplex *plus* HRP. In the presence of TEGDMA (5 mM) H_2O_2 production was stimulated by 56% (from 369 ± 8 to 576 ± 12 pmol/min/mg protein) (Fig. 4A). Addition of ADP decreased the rate of H_2O_2 formation, but in the presence of TEGDMA 95% stimulation was detected.

3.3.3. *H_2O_2 elimination in mitochondria supported by NADH-linked substrates*

Mitochondria were challenged with exogenous H_2O_2 in the presence or absence of TEGDMA (5 mM) and the disappearance of H_2O_2 was tested as described in Section 2. In the presence of TEGDMA a significant decrease in H_2O_2 elimination was observed (Fig. 4B).

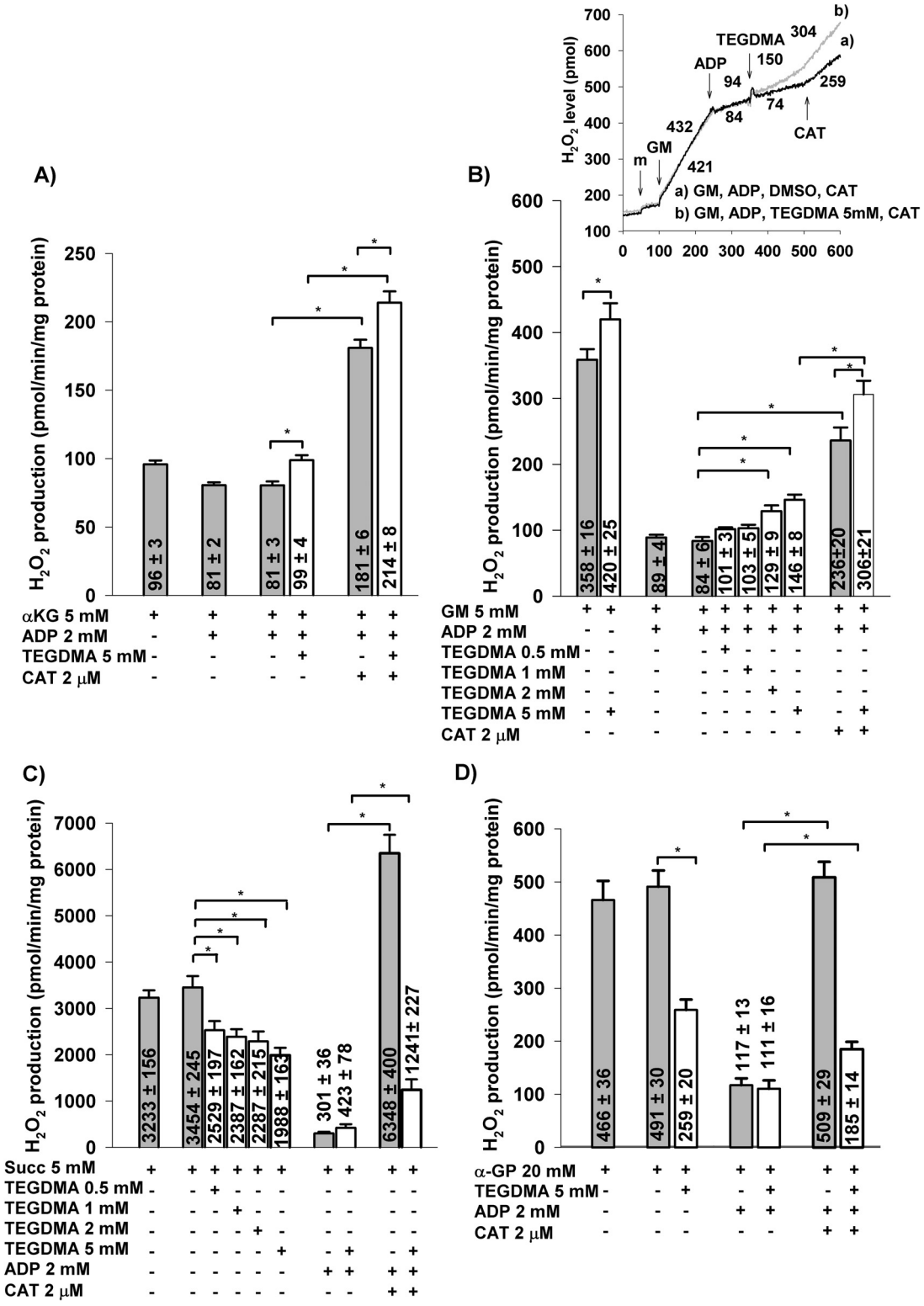

Fig. 3 – Effects of TEGDMA on H_2O_2 production in the isolated brain mitochondria supported by α-ketoglutarate (α-KG; A), glutamate *plus* malate (GM; B), succinate (Succ; C), or α-glycerophosphate (20 mM) (α-GP; D). In the inset of B, original traces and numbers that represent the rates of H_2O_2 production (in pmol/min/mg protein) are displayed. Carboxyatractylate (CAT) was added as indicated to the mitochondria incubated with GM, ADP, TEGDMA (*gray curve*), or DMSO (*black curve*) (*Inset B*). For A–D, the rates of H_2O_2 production were also demonstrated on a bar chart (mean ± SEM; *white bars*: TEGDMA was present, *grey bars*: no TEGDMA was present). Statistical analysis was applied as above (see Fig. 1).

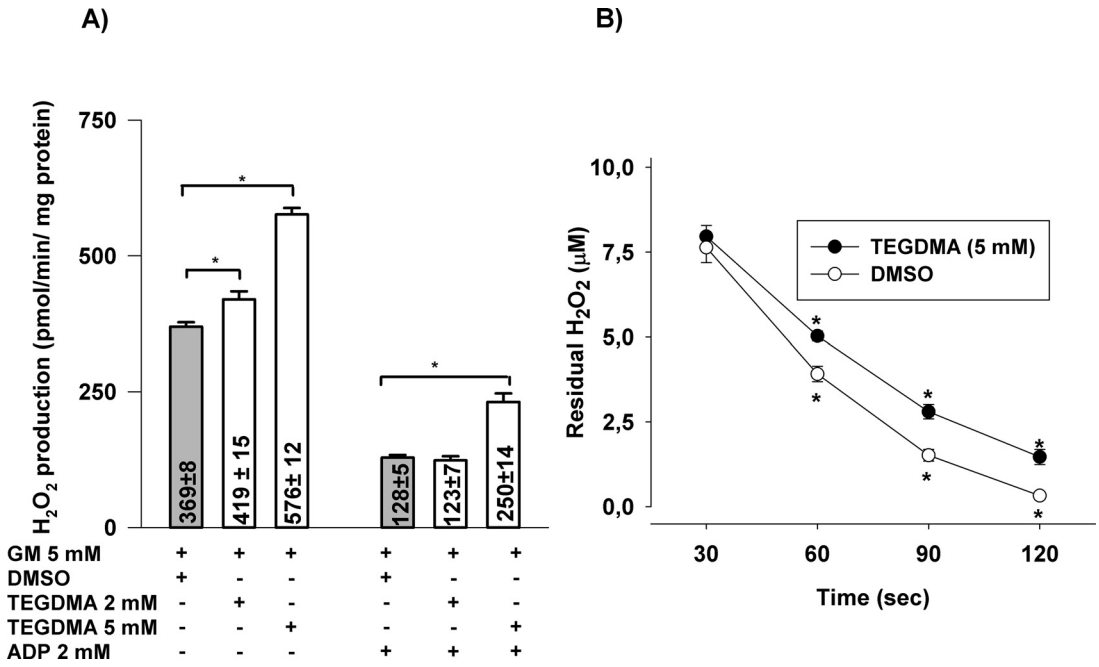

Fig. 4 – Effects of preincubation of mitochondria with TEGDMA on H_2O_2 production (A), and H_2O_2 elimination (B). Mitochondria were preincubated for 10 min with Complex I substrate glutamate *plus* malate (5–5 mM) in the incubation medium in the absence (*grey bars*) or presence of TEGDMA (*white bars*). After this 10 min, horseradish peroxidase and Amplex Ultra were added (as described in Section 2) and H_2O_2 production was measured. Data are expressed as mean ± SEM (n ≥ 7) (A). Removal of exogenously added H_2O_2 by glutamate *plus* malate supported mitochondria in the presence or absence of TEGDMA (5 mM). Mitochondria (0.1 mg/ml) were incubated for 10 min, then challenged with H_2O_2 (10 μM). The residual H_2O_2 was measured from aliquots taken after different time intervals, as described in Section 2. Data are expressed as mean ± SEM (n = 9) (B). Significant results ($p < 0.05$) are indicated by asterisks (A and B).

3.3.4. *The interaction of TEGDMA with GSH*

Similarly to the experiments described elsewhere [52], the reaction of GSH with TEGDMA (5 mM) was investigated in a mitochondrion-free reaction medium. At 15 min of incubation time a 3.7% decrease in GSH concentration was detected in the presence of TEGDMA (5 mM) (Fig. 5).

3.3.5. *H_2O_2 formation in the courses of oxidation of non-Complex I substrates*

3.3.5.1. *H_2O_2 generation in mitochondria supported by succinate.* TEGDMA or DMSO were applied to mitochondria energized with succinate. TEGDMA (5 mM) decreased the rate of H_2O_2 production by 42% (from 3454 ± 245 to 1988 ± 163 pmol/min/mg protein). Significant decrease of H_2O_2 formation has been observed already at 0.5 mM of TEGDMA. Addition of ADP further decreased H_2O_2 production to 423 ± 78 pmol/min/mg protein in the presence of TEGDMA (5 mM) and to 301 ± 36 pmol/min/mg protein in DMSO-treated mitochondria (Fig. 3C). Inhibition of ANT by CAT stimulated again the ROS production. H_2O_2 generation in TEGDMA-treated group (1241 ± 227 pmol/min/mg protein) was significantly lower than that of control group (6348 ± 400 pmol/min/mg protein) (Fig. 3C) owing to the CI inhibition.

3.3.5.2. *H_2O_2 formation in mitochondria supported by α-GP.* In brain mitochondria alpha-glycerophosphate dehydrogenase (α-GPDH) is highly active [53]. This enzyme (such as succinate dehydrogenase) provides the respiratory chain with electrons and can also generate RET. In α-GP-energized mitochondria TEGDMA mediated a 47% suppression of H_2O_2 production (from 491 ± 30 to 259 ± 20 pmol/min/mg protein) (Fig. 3D). Addition of ADP lowered the rate of H_2O_2 generation in both groups. In the presence of ADP there was no observable difference between the two treatments (DMSO or TEGDMA). Under control conditions CAT restored the rate of H_2O_2 production to the level detected in the ADP-free state (from 117 ± 13 to 509 ± 29 pmol/min/mg protein). In TEGDMA-treated mitochondria there was also a CAT-mediated stimulation of H_2O_2 generation (from 111 ± 16 to 185 ± 14 pmol/min/mg protein), but the effect was much smaller than the one measured in solvent-treated mitochondria (Fig. 3D).

3.4. *Effects of TEGDMA on the NAD(P)H level*

The application of both NADH-linked (α-KG, GM) and $FADH_2$-linked substrates (Succ and α-GP) elevated the mitochondrial NAD(P)H level (Fig. 6). The elevation of the NAD(P)H level by Succ and/or α-GP can likely be ascribed to the RET mechanism [54–56]. The ADP-induced stimulation of respiration was accompanied by a decreased steady-state level of NAD(P)H. In TEGDMA-treated mitochondria the NAD(P)H steady-state level was higher both with either NADH or $FADH_2$-linked substrates (Fig. 6 *traces b*). In mitochondria respiring on α-KG the

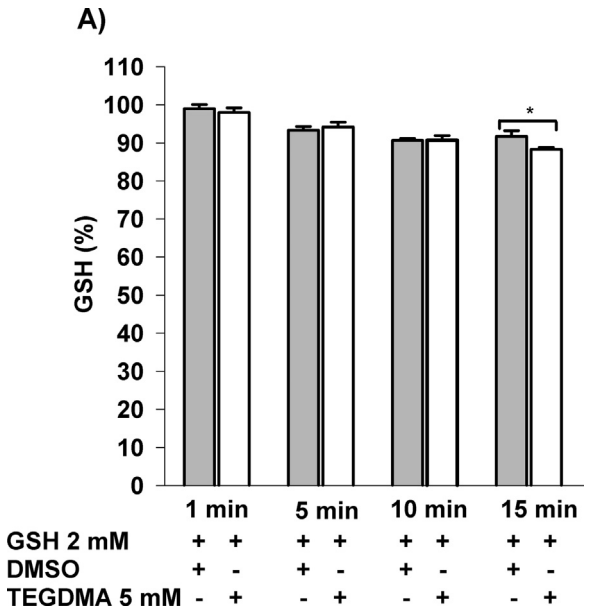

Fig. 5 – Effect of incubation with TEGDMA on the GSH level in mitochondrion-free medium. GSH (2 mM) was incubated with TEGDMA (5 mM) in the standard incubation medium and at the time intervals indicated aliquots were taken and the reduced glutathione concentration was determined by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), spectrophotometrically ($\varepsilon_{412} = 13600\ M^{-1}\ cm^{-1}$). Results are expressed as percentage of original GSH concentration; mean ± SEM ($n \geq 7$). Significance ($p < 0.05$) is indicated by asterisk.

TEGDMA-induced elevation of the NAD(P)H level was slow and gradual (Fig. 6A), but in GM-supported mitochondria TEGDMA induced a fast elevation of the NAD(P)H level (Fig. 6B). In the presence of CAT there was no difference in the NAD(P)H steady state between control and TEGDMA challenged mitochondria (Fig. 6). However, in TEGDMA-treated succinate-energized mitochondria ADP did not decrease the NADP(H) level at all, contrary to TEGDMA-treated α-GP-supported mitochondria where a moderate decrease of the NAD(P)H level was observed (Fig. 6C,D *traces b*).

3.5. *Effect of TEGDMA on ATP synthesis*

For a better characterization of the relevant mitochondrial bioenergetics, in addition to oxygen consumption and $\Delta\psi_m$ the rate of ATP production was also assessed (Fig. 7). After administering ADP, in the absence of respiratory substrates, a compromised rate of the adenylate kinase (AK) coupled ATP synthesis (18 ± 2 nmol/min/mg protein) could still be detected as 200 μM AP5 was apparently not sufficient to fully inhibit the activity of AK. OXPHOS was initiated with the addition of respiratory substrates in the presence of ADP. In GM-supported TEGDMA-treated mitochondria the rate of ATP production was lowered by 86% (decreased from 824 ± 23 to 113 ± 13 nmol/min/mg protein) as compared with controls. However, addition of TEGDMA (5 mM) to succinate-energized mitochondria resulted in a drop of 10.2% only in the rate of ATP synthesis (from 561 ± 24 to 504 ± 25 nmol/min/mg protein).

3.6. *The effect of TEGDMA on Complex I activity*

In order to prove our hypothesis that TEGDMA was an inhibitor of CI, the NADH-CoQ reductase activity was directly measured. The CI activity was decreased by 70.6 ± 3.3 and 61.8 ± 3.2% in the presence of 5 and 2 mM TEGDMA, respectively (data not shown).

4. Discussion

It has been shown that TEGDMA release due to incomplete polymerization and biodegradation in oral environment can impair various cellular functions, provoke oxidative stress (for review see Ref. [18]), apoptotic and necrotic cell death [28,30,57,58] and trigger genetic mutations [8,59]. Selected resin monomers were demonstrated to deplete the pool of glutathione (GSH), an intracellular antioxidant [12]. Inhibition of cell proliferation, stimulation of apoptosis and impaired differentiation of osteoclasts [60] can be all mediated by the overproduction of ROS. The beneficial effects of antioxidants *e.g.* *N*-acetylcysteine (NAC), Trolox (vitamin E), or ascorbic acid (vitamin C) also suggest that the cytotoxic effects of TEGDMA would be the consequences of increased ROS generation [20,57,61–65]. The cellular mechanism that lies behind the increased rate of ROS production has not been elucidated yet [18]. The involvement of the mitochondrion in TEGDMA toxicity has already been proposed [32]. A recent study emphasized the importance of the TEGDMA-mediated activation of NOX4 in the ROS generation and apoptosis [31]. Since most of the ATP, required for various cellular functions, is produced by mitochondria, this organelle plays a pivotal role in cellular viability and death. The mitochondrion is also a primary site for ROS production and elimination (for review, see Ref. [38]). Therefore, we investigated the responses of various mitochondrial functions to TEGDMA.

4.1. *Effects of TEGDMA on mitochondrial respiration*

Mitochondrial respiration is a sensitive indicator of mitochondrial function [66]. In mitochondria which were supported by either α-KG or GM TEGDMA (5 mM) led to a decrease in oxygen consumption (Fig. 1A–C), whereas in $FADH_2$-linked, succinate-supported mitochondria the rate of oxygen consumption was not affected by this treatment (Fig. 1D). Our results are in contrast with an earlier observation [67] where the respiration of isolated succinate-rotenone-supported mitochondria was inhibited by TEGDMA; this contradiction might be due to different experimental setups. In the above paper HL-60 cells were preincubated with TEGDMA for 1 h, and mitochondria were isolated and investigated thereafter. In the course of the incubation period mitochondria might have undergone such a structural damages that could potentially also impair the succinate-dependent respiration. In the present study, the reduced rates of mitochondrial respiration observed in the presence of CI substrates can potentially be explained by: (i) a decreased ATPase activity [68–70], (ii) the inhibition of ANT

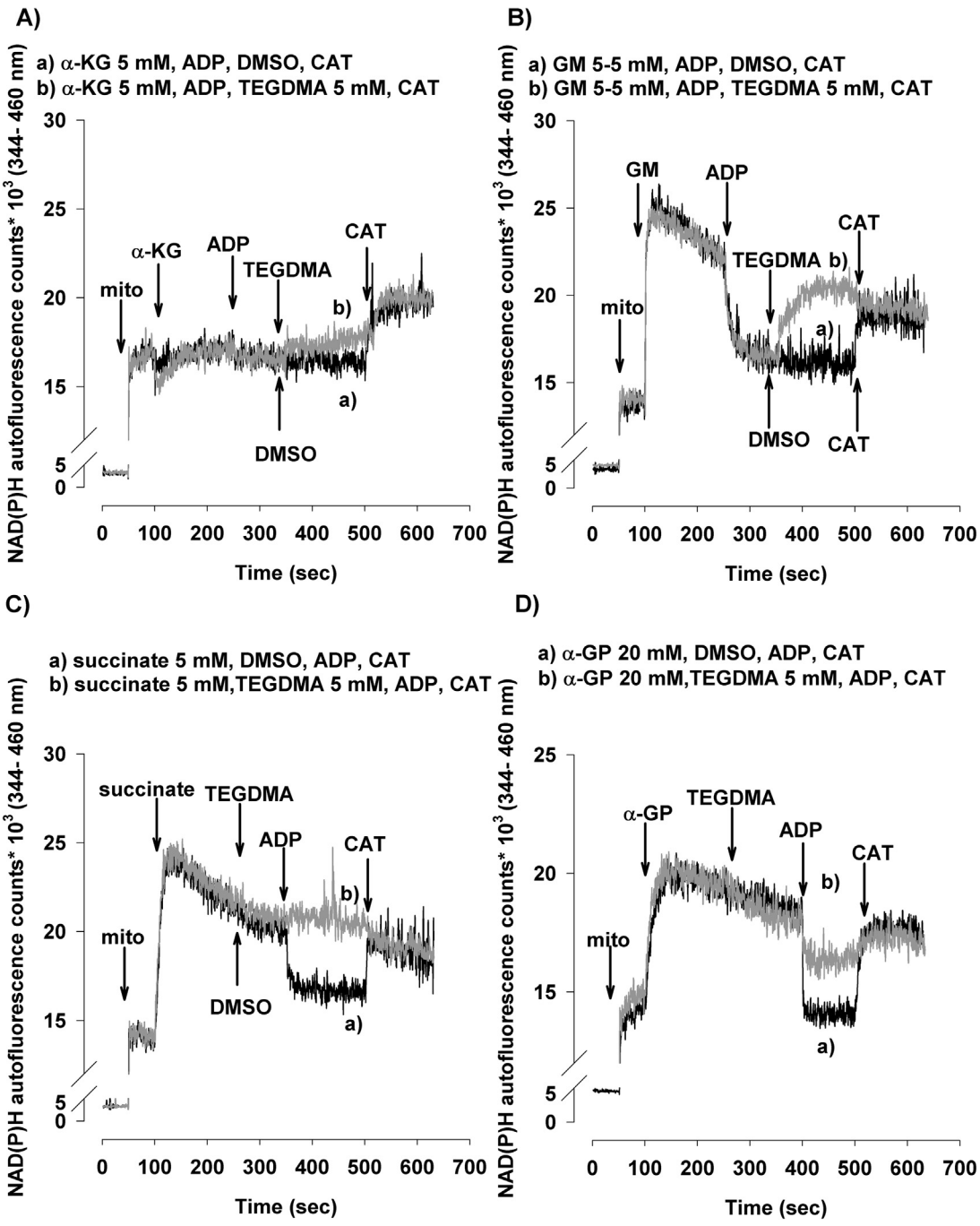

Fig. 6 – Effects of TEGDMA on the steady-state level of NAD(P)H in the brain mitochondria supported by α-ketoglutarate (A), glutamate *plus* malate (B), succinate (C), or α-glycerophosphate (D). Mitochondria (0.1 mg/mL) were incubated with various respiratory substrates. TEGDMA or DMSO, ADP (2 mM), and carboxyatractylate (CAT, 2 μM) were added as indicated. Representative original traces of experiments are displayed (*grey, trace b*: TEGDMA was present; *black, trace a*: no TEGDMA was present).

[71], (iii) a reduced rate of transport for metabolites and/or inorganic phosphate [72], or (iv) the compromised activities of CI and/or selected dehydrogenase enzymes [73,74]. However, the normal rate of respiration which was detected in the presence of succinate excluded the reasons listed under i, ii, and partially iii. In order to elucidate the mechanism that lies behind the depression of respiration the effects of TEGDMA on mitochondrial $\Delta\psi_m$ were assessed.

4.2. *Effects of TEGDMA on mitochondrial* $\Delta\psi_m$

Maintenance of the mitochondrial membrane potential is crucial for normal mitochondrial physiology. The proton gradient is used by mitochondria to facilitate transport processes for ions [75], metabolites [76] and proteins [77] across the mitochondrial inner membrane. Previously, $\Delta\Psi_m$ was measured in gingival fibroblasts and mitochondrial depolarization

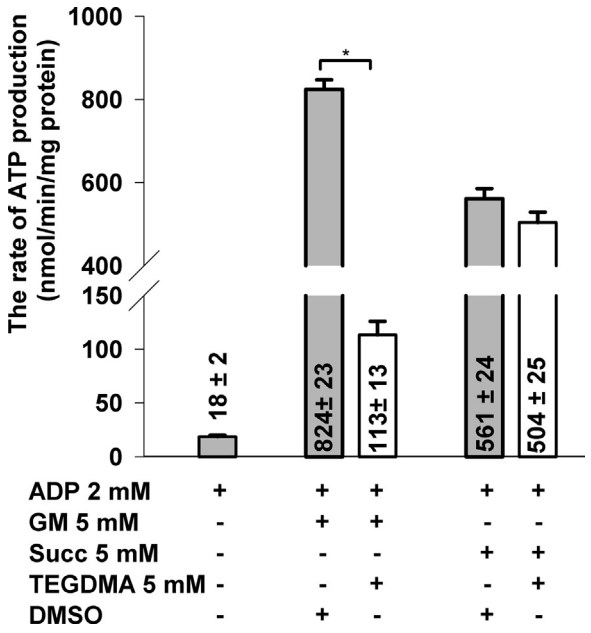

Fig. 7 – Effects of TEGDMA on the rate of ATP synthesis in brain mitochondria respiring on either glutamate *plus* malate or succinate. TEGDMA (*white bars*) or DMSO (*grey bars*) were applied besides ADP throughout all the experiments. The rate of ATP synthesis, written on the bars, is expressed in nmol/min/mg protein (mean ± SEM) n > 11. Significant differences are indicated by $^{*}p < 0.05$.

was detected [32]. In cellular systems, however, mitochondria are energized simultaneously with various respiratory substrates and hence it is difficult to determine the true target of a compound. In the present study, $\Delta\Psi_m$ measurements also confirmed that TEGDMA inhibits the CI. In mitochondria supported with NADH-linked substrates, addition of TEGDMA decreased $\Delta\Psi_m$ (Fig. 2A,B). Contrary to that, in succinate-energized mitochondria $\Delta\Psi_m$ was unaffected by TEGDMA (Fig. 2C). These findings again prove that TEGDMA does not inhibit the ANT or the ATP-ase; when using selective inhibitors of these molecules $\Delta\Psi_m$ would have been hyperpolarized, independently of the respiratory substrates applied [78]. The results are consistent with the proposed inhibitory effect of TEGDMA on the CI function.

4.3. *Effects of TEGDMA on ROS homeostasis*

As we discussed earlier, there is a general agreement that exposition of cells to TEGDMA provokes oxidative stress. However, the sources and mechanisms of the redox imbalance have not been elucidated, yet. Very recently it was suggested that the activation of NADPH oxidase 4 has a key role in the TEGDMA-associated ROS generation [31].

Our hypothesis that mitochondrial targets are also involved in the cellular toxicity of TEGDMA would be substantiated if administration of TEGDMA could stimulate the mitochondrial ROS production.

4.3.1. *H_2O_2 formation with respiratory substrates linked to Complex I*

The addition of respiratory substrates to mitochondria initiated mitochondrial H_2O_2 generation (Fig. 3). Both α-KG and GM stimulated the H_2O_2 production, but to different extent (Fig. 3A,B). The higher rate of H_2O_2 production with GM can be ascribed to the higher rate of GM-induced respiration (Fig. 1C) and to the consequently higher $\Delta\psi_m$ (Fig. 2B) [51]. There is a direct correlation between the membrane potential and H_2O_2 production (Figs. 2A,B and 3A,B) and Starkov et al. [51]. Addition of ADP differently inhibited H_2O_2 formation on α-KG and GM respiring mitochondria (Fig. 3A,B). The higher was the membrane potential before the ADP addition, the greater was the decrease of H_2O_2 production (Fig. 3A,B). Taking into consideration that the α-KGDH is the rate-limiting (slowest) enzyme of the citric acid cycle in brain mitochondria, it is obvious that the respiration rate, as well as the rate of H_2O_2 production, is slower with α-KG than with GM. When it was given after ADP, TEGDMA significantly augmented the rate of H_2O_2 production and this was valid under both experimental conditions (with α-KG: 18% increase, with GM: 74% increase, relative to controls; see Fig. 3A,B). Elevated rates of H_2O_2 production were observed in the presence of CI inhibitors; CI possesses several sites that are capable of producing ROS (for review see Ref. [79]). In the electron transport chain, those electron carrier complexes that are upstream to the binding site of a respiratory chain inhibitor are in highly reduced states [40]. Reduction of a ROS-forming site increases the likelihood for an electron leakage. Inhibition of CI in CI substrate (glutamate *plus* malate) supported mitochondria stimulated H_2O_2 formation in either the presence or absence of ADP. Enhanced H_2O_2 production therefore can deplete endogenous antioxidants such as glutathione and inhibit glutathione-related antioxidant systems [80].

Comparing the results depicted in Figs. 3B and 4A, it is apparent that as a consequence of the Amplex *plus* HRP-free preincubation the stimulatory effect of TEGDMA on H_2O_2 production was further enhanced in either the presence or absence of ADP. We interpret these results that continuous monitoring of mitochondrial H_2O_2 production with Amplex *plus* HRP not only detects, but also detoxifies the generated H_2O_2 [44] mitigating the oxidative damage.

Considering that mitochondria play roles in both ROS formation and ROS elimination, the effect of TEGDMA on the elimination of exogenous H_2O_2 was also investigated. Our results indicate (Fig. 4B) that in the presence of TEGDMA the elimination was indeed slower, therefore these results further confirm the hypothesis drawn from the preincubation experiment that TEGDMA can impair the antioxidant system. This phenomenon can also be attributed to the inhibition of Complex I, which results in a decrease in the mitochondrial membrane potential. $\Delta\psi_m$ is a driving force for the energy-dependent transhydrogenase enzyme that can transfer electrons from NADH to $NADP^+$ [81], thus can maintain the NADPH level, which is necessary for the regeneration of GSH.

However, it is still obscure whether TEGDMA can directly interfere with the antioxidant system. In the excellent paper of Lefeuvre et al. [52] the direct effect of TEGDMA on GSH has been investigated and it was found that incubation of

TEGDMA (3 mM) with GSH decreased the GSH concentration in the absence of cells. After 1 and 3 h of incubation significant decrease of [GSH] was detected. We reinvestigated this question and using 5 mM TEGDMA significant changes were found after 15 min of incubation, 3.7% decrease of [GSH] was detected (Fig. 5). According to Lefeuvre et al. incubation gingival fibroblasts with low concentration of TEGDMA (0.3 mM) generated a high rate of GSH disappearance, at 30 min GSH level decreased by more than 50%. Comparison of *in vitro* and cellular experiments suggest that the decrease of GSH level is not a consequence of direct interaction with TEGDMA. In Lefeuvre's paper the possible role of glutathione-S-transferase was put forward. The results of the present paper suggest an additional target – mitochondria – for TEGDMA's action. The suggested mechanisms for TEGMA's cytotoxicity do not exclude each other.

4.3.2. *H_2O_2 production with $FADH_2$-linked substrates (Succ and α-GP) and the effect of inhibiting the reverse electron transport*

From $FADH_2$ linked respiratory substrates most of the electrons are transported to oxygen as the final electron acceptor *via* the Complexes III and IV. It is well documented however, that with succinate in the absence of ADP a high $\Delta\psi_m$ is generated that drives a certain flux of electrons from coenzyme Q (CoQ) to flow towards the CI, in a direction that is inverse to normal. This phenomenon is referred to as the reverse electron transport (RET) mechanism [82,83] that results in the conversion of NAD^+ to NADH at the CI. RET is the predominant ROS-generator when no ADP is present and the mitochondria are supported by either succinate or α-GP [40,53]. TEGDMA lowered the rate of the mitochondrial H_2O_2 production by 42% (for Succ) or 47% (for α-GP) (Fig. 3C,D). This seemingly paradoxical phenomenon can be attributed to a contribution by the so-called reverse electron transport (RET) mechanism, a backward flow of electrons from the reduced $CoQH_2$ towards the CI (and NAD^+) [50,84–88]. CAT hyperpolarized $\Delta\psi_m$ and therefore restored the RET in the DMSO-treated control group. In the TEGDMA-treated mitochondria, however, H_2O_2 production was less efficient due to inhibition of the CI (Fig. 3C,D).

4.4. *Relationship between inhibition of Complex I and the NAD(P)H level in TEGDMA-treated mitochondria*

Complex I substrates (α-KG, GM) led to NADH formation (Fig. 6A,B), because these substrates are metabolized by the dehydrogenases to yield NADH. In the presence of ADP most of the NADH became oxidized, however, addition of TEGDMA increased the steady state level of NADH/NAD (Fig. 6A,B). This observation is crucial since it implies that TEGDMA does inhibit the CI, but does not compromise the related dehydrogenases. With non-Complex I substrates (*e.g.* Succ, α-GP) the formation of NADH can unambiguously be attributed to RET as the respective dehydrogenases do not produce NADH. Addition of ADP to energized mitochondria decreased the steady state level of NADH for all the tested respiratory substrates (Fig. 6A–D), because (i.) ADP stimulates the respiration, (ii.) a higher rate of the electron flow promotes NADH oxidation, and (iii.) an ADP-induced decrease in the membrane potential abolishes the reverse electron flow [89]. TEGDMA induced different responses in mitochondria depending on the substrate used for energization. With CI substrates TEGDMA-mediated inhibition of the Complex I withheld electrons from reaching the distal sequences of respiratory chain; consequently, NAD^+ mostly got reduced to NADH (Fig. 6A,B). In the presence of succinate or α-GP the addition of TEGDMA preceded the administration of ADP to mitochondria (Fig. 6C,D). In succinate-supported mitochondria addition of ADP was unable to drop NADH concentration to the control level, because CI was inhibited, thus the accumulated NADH was unable to be oxidized (Fig. 6C). For more detailed explanation see also Ref. [44].

4.5. *ATP production in TEGDMA-challenged mitochondria*

In order to minimize the contribution of the adenylate kinases (AKs) to the mitochondrial ATP generation the AK inhibitor 2-amino-phoshponophentanoate (AP5) [90] was applied in the experiments that assessed ATP production. In the presence of ADP and the absence of any respiratory substrates only the AK-related ATP synthesis could be detected; the AK activity was only partially inhibited. Addition of the respiratory substrate (GM or Succ) led to the initiation of OXPHOS. In GM-supported mitochondria TEGDMA lowered the rate of ATP synthesis by 86% (Fig. 7). This observation suggests that TEGDMA, which was inhibiting the CI, caused depolarization and the remaining proton gradient was not sufficient to support a high rate of OXPHOS; compromised ATP synthesis leads to cellular bioenergetic insufficiency and eventually necrotic cell death.

4.6. *Complex I activity in TEGDMA-treated mitochondria*

The direct measurement of CI activity proved that TEGDMA can indeed inhibit the CI. The immediate effect of TEGDMA suggests that CI can in fact be a primary target of this resin monomer. In rotenone-treated (CI inhibitor) cells oxidative stress, glutathione depletion, and DNA damage were detected with a subsequently increased prevalence of apoptosis [80]. It has been described that various inhibitors of the CI are able to stimulate the mitochondrial ROS production and cause MAPK/NF-κB activation [91]. Furthermore, the extent of CI inhibition can determine the type of cell death induced. Low doses of CI inhibitors generally induce apoptosis, while higher doses of the same compound may evoke necrotic cell death [37].

Our study focused on the acute effects of TEGDMA and the results do not contradict the observations made by Yeh et al. [31]. The development of TEGDMA-induced changes which were attributed to NOX4 activation required hours and changes in the gene expression of NOX4 were observed [31]. Recently, it was reported that inhibition of the CI by rotenone can stimulate NOX2 expression [92]. Thus, we propose that the CI is an immediate target of TEGDMA and that the activation of various signal transduction mechanisms may be required to activate the NOX isoenzymes.

The effects and consequences of inhibitory action of TEGDMA on respiratory Complex I are summarized on Fig. 8.

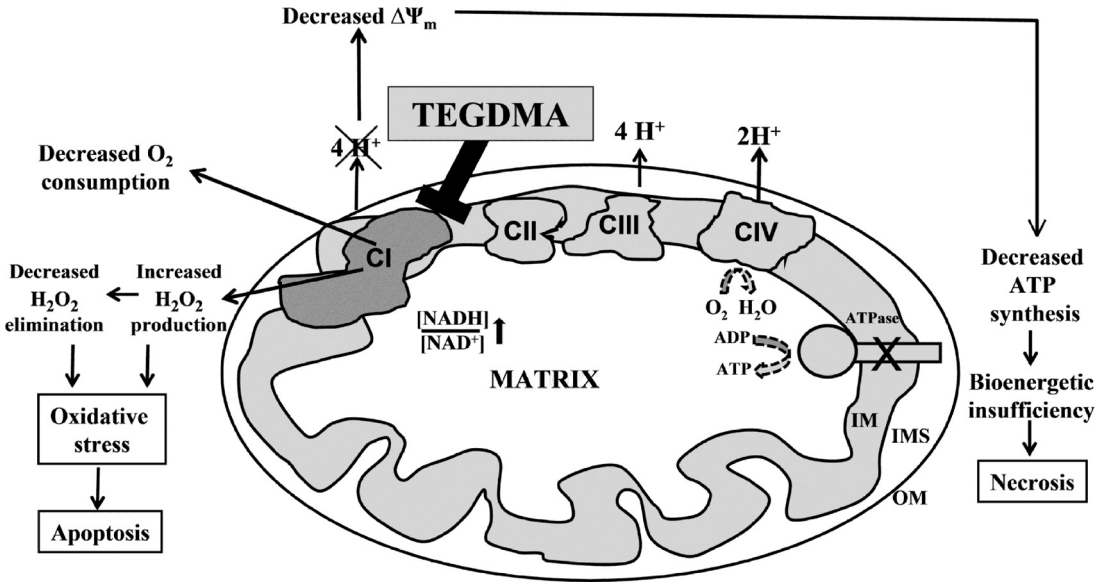

Fig. 8 – Schematic summary of TEGDMA-induced mitochondrial damage. The primary target of TEGDMA is identified as the CI. The effects of CI inhibition are decreased O_2 consumption, stimulation of H_2O_2 production, impairment of mitochondrial H_2O_2 elimination with a consequent oxidative stress, and apoptosis. The decrease of ATP synthesis at high TEGDMA concentrations can create necrotic type of cell death. For simplicity the reverse electron flow (see Section 4.3.2) is not indicated on the figure. Abbreviations: IM: inner mitochondrial membrane; IMS: intermembrane space; OM: outer membrane; CI, II, III, IV: respiratory complexes, $\Delta\psi_m$: mitochondrial membrane potential.

5. Conclusions

We identified the CI of the mitochondrial electron transport chain as a target of TEGDMA resin monomer. Addition of TEGDMA results in inhibition of the CI-dependent oxygen consumption, depolarization of mitochondrial membrane, and consequently decrease in ATP production *via* OXPHOS, therefore TEGDMA causes bioenergetic insufficiency. Inhibition of CI by TEGDMA enhances the mitochondrial H_2O_2 (ROS) formation and impairs ROS elimination. The direct interaction between TEGDMA and reduced glutathione is unlikely to be a major component in the toxicity.

Acknowledgments

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