The Neuromediator Glutamate, through Specific Substrate Interactions, Enhances Mitochondrial ATP Production and Reactive Oxygen Species Generation in Nonsynaptic Brain Mitochondria*

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The finding that upon neuronal activation glutamate is transported postsynaptically from synaptic clefts and increased lactate availability for neurons suggest that brain mitochondria (BM) utilize a mixture of substrates, namely pyruvate, glutamate, and the tricarboxylic acid cycle metabolites. We studied how glutamate affected oxidative phosphorylation and reactive oxygen species (ROS) production in rat BM oxidizing pyruvate + malate or succinate. Simultaneous oxidation of glutamate + pyruvate + malate increased state 3 and uncoupled respiration by 52 and 71%, respectively. The state 4 ROS generation increased 100% over BM oxidizing pyruvate + malate and 900% over that of BM oxidizing glutamate + malate. Up to 70% of ROS generation was associated with reverse electron transport. These effects of pyruvate + glutamate + malate were observed only with BM and not with liver or heart mitochondria. The effects of glutamate + pyruvate on succinate-supported respiration and ROS generation were not organ-specific and depended only on whether mitochondria were isolated with or without bovine serum albumin. With the non-bovine serum albumin brain and heart mitochondria oxidizing succinate, the addition of pyruvate and glutamate abrogated inhibition of Complex II by oxaloacetate. We conclude that (i) during neuronal activation, simultaneous oxidation of glutamate + pyruvate temporarily enhances neuronal mitochondrial ATP production, and (ii) intrinsic inhibition of Complex II by oxaloacetate is an inherent mechanism that protects against ROS generation during reverse electron transport.

Recently, it has emerged that mitochondrial dysfunctions play an important role in the pathogenesis of degenerative diseases of the central nervous system (1–3). The processes underlying neuronal degeneration are complex, and some authors suggest that several genetic alterations are involved (4). However, another level of complexity may be derived from the fact that virtually all cellular activities depend upon energy metabolism in the cell (5). Alterations in energy metabolism processes within cells may also contribute to pathogenic mechanisms underlying neurodegenerative disease.

A large body of evidence suggests that increased oxidative stress is an important pathogenic mechanism that promotes neurodegeneration (6). Because neurons have a long life span, and most neurodegenerative diseases have a clear association with age (7), it is important to understand mechanisms underlying reactive oxygen species (ROS) production in neurons. Recently, Kudin et al. (8) analyzed the contribution of mitochondria to the total ROS production in brain tissue. They concluded that mitochondria are the major source of ROS and that at least 50% of ROS generated by brain mitochondria was associated with succinate-supported reverse electron transport (RET). Under conditions of normoxia, about 1% of the respiratory chain electron flow was redirected to form superoxide (8).

Recently, we suggested that the organization of the respiratory chain complexes into supercomplexes that occurs in brain mitochondria (BM) (9) may represent one of the intrinsic mechanisms to prevent excessive ROS generation (10). In this paper, we put forward the hypothesis that inhibition of Complex II by oxaloacetate (OAA) represents another important intrinsic mechanism to prevent oxidative stress. We provide evidence that glutamate and pyruvate specifically exert control over the production of ROS at the level of Complex II. Below we present a brief account of published theoretical and experimental evidence that underlie our hypothesis.

The neural processing of information is metabolically expensive (11). More than 80% of energy is spent postsynaptically to restore the ionic composition of neurons (11). When neurons are activated, reuptake of glutamate stimulates aerobic glycolysis in astroglial cells (12), thereby making lactate the major substrate for pyruvate transport into brain mitochondria (BM) (9). We suggest that the brain is unique in this respect due to the large amount of energy needed to support brain function (11). The brain relies on oxidative phosphorylation to produce ATP, but at the same time, it consumes large amounts of ROS, which are generated during mitochondrial respiration (11).

The abbreviations used are: ROS, reactive oxygen species; AGC, aspartate-glutamate carrier; BM, brain mitochondria; BSA, bovine serum albumin; CCCP, cyanide-chloropheynylhydrazione; α-KG, α-ketoglutarate; α-KGDHC, α-ketoglutarate dehydrogenase complex; MAS, malate aspartate shuttle; MOPS, 4-morpholinepropane sulfonic acid sodium salt; OAA, oxaloacetate; RET, reverse electron transport; SDH, succinate dehydrogenase; TPP †, tetraphenyl phosphonium; RHM, rat heart mitochondria; non-BSA-BM, BM isolated in the absence of BSA.

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2 The abbreviations used are: ROS, reactive oxygen species; AGC, aspartate-glutamate carrier; BM, brain mitochondria; BSA, bovine serum albumin; CCCP, cyanide-chloropheynylhydrazione; α-KG, α-ketoglutarate; α-KGDHC, α-ketoglutarate dehydrogenase complex; MAS, malate aspartate shuttle; MOPS, 4-morpholinepropane sulfonic acid sodium salt; OAA, oxaloacetate; RET, reverse electron transport; SDH, succinate dehydrogenase; TPP †, tetraphenyl phosphonium; RHM, rat heart mitochondria; non-BSA-BM, BM isolated in the absence of BSA.
substrate for neuronal mitochondria (4, 13). However, rapid conversion of lactate to pyruvate in neurons requires activation of the malate-aspartate shuttle (MAS). The shuttle is the major pathway for cytosolic reducing equivalents from NADH to enter the mitochondria and be oxidized (14, 15). The key component of MAS is the mitochondrial aspartate/glutamate carrier (AGC) (16), and recent data suggest that the AGC is expressed mainly in neurons (14). Absence of the AGC from astrocytes in the brain implies a compartmentation of intermediary metabolism, with glycolysis taking place in astrocytes and lactate oxidation in neurons (13, 14, 17). Active operation of MAS requires that a certain amount of glutamate must be transported from synaptic clefts into activated neurons. In isolated BM, it has been shown that besides pyruvate, glutamate is also a good respiratory substrate (5, 18). In the presynaptic elements, the concentration of cytosolic glutamate is ~10 mM at all times (19). Yudkoff et al. (18) have shown that synaptosomal mitochondria utilize glutamate and pyruvate as mitochondrial respiratory substrates. Glutamate is also oxidized by the astroglial mitochondria (13).

Until recently, it was generally accepted that most of the glutamate is rapidly removed from the synaptic cleft by glutamate transporters EAAT1 and EAAT2 located on presynaptic termini and glial cells (20–24). However, recent data show that a significant fraction of glutamate is rapidly bound and transported by the glutamate transporter isoform, EAAT4, located juxtasynaptically in the membranes of spines and dendrites (20, 25–28). At the climbing fiber to Purkinje cell synapses in the cerebellum, about 17% (28) or more than 50% (29) of synthetically released glutamate may be removed by postsynaptic transporters. Besides the cerebellum, EAAT4 protein was found to be omnipresent throughout the fore- and midbrain regions (30). Moreover, it was shown that although most of the EAAT2 protein is astroglial, around 15% is distributed in nerve terminals and axons in hippocampal slices and that this protein may be responsible for more than half of the total uptake of glutamate from synaptic clefts (24). These data suggest that postsynaptic transport of glutamate into nerve terminals where mitochondria are located (31) may occur in all brain regions. According to calculations of Brasnjo and Otis (28), in a single synapse, EAAT4 (excitatory amino acid transporter 4) binds and transports postsynaptically about 1.3 ± 0.1 × 10⁶ glutamate molecules. In the brain, on average, 1 mm² of tissue contains 1 × 10⁸ synapses (32, 33). Because of the high density of synaptic contacts, the neuronal cells may be exposed to mediators released from hundreds of firing synapses. Thus, in a narrow space of spines and dendrites, several million glutamate molecules postsynaptically transported from synaptic boutons may create local cytosolic concentration of glutamate in the low millimolar range. Consequently, neuronal mitochondria, particularly those located at the axonal or dendritic synaptic junctions, may, in addition to metabolizing pyruvate, temporarily metabolize glutamate and succinate formed during mitochondrial catabolism of γ-aminobutyric acid in postsynaptic cells (34).

The purpose of this study was to examine how the neuromediator glutamate affects respiratory activity and ROS generation in nonsynaptic BM when combined with pyruvate and the tricarboxylic acid cycle intermediates succinate and malate. We show that with pyruvate + glutamate + malate, the rate of oxidative phosphorylation increased more than 50%, and in resting mitochondria the rate of ROS generation associated with the reverse electron transport increased severalfold. These effects were observed only with brain and spinal cord mitochondria, not with liver or heart mitochondria, suggesting that they may be restricted to neuronal cells.

Taken together, the data presented support the hypothesis that in activated neurons, the neuromediator glutamate stimulates mitochondrial ATP production when energy demand is increased. However, in the absence of energy consumption, glutamate + pyruvate may increase the generation of ROS severalfold. We suggest that intrinsic inhibition of Complex II by oxaloacetate is an important natural protective mechanism against ROS associated with reverse electron transport.

**EXPERIMENTAL PROCEDURES**

**Animals**—All animal use complied with National Institutes of Health guidelines and was approved by the Institutional Animal Care and Use Committee of the Carolinas Medical Center. Male Sprague-Dawley rats (180–250 g) from Taconic Farms Inc. (Germantown, NY) were used for isolation of BM.

**Isolation of Mitochondria**—Brain mitochondria were isolated from pooled forebrains of three rats using a modified method of Sims (37), as described in Ref. 10. Rat heart and liver mitochondria were isolated as described in Refs. 10 and 38. The isolation medium contained 75 mM mannitol, 150 mM sucrose, 20 mM MOPS, pH 7.2, and 1 mM EGTA. For experiments examining effects of defatted bovine serum albumin (BSA), 0.1% BSA was added to the isolation medium. The final suspensions of mitochondria were prepared using a medium containing 75 mM KCl, 125 mM succrose, and 10 mM MOPS. Mitochondrial protein was determined with the Pierce Coomassie protein assay reagent kit.

**Simultaneous Measurement of Mitochondrial Respiration and Membrane Potential**—Respiratory activities of the mitochondria were measured using a custom-made plastic minichamber of 560 μl volume equipped with a standard YSI (YSI Inc.) oxygen minielectrode connected to a YSI model 5300 Biological Oxygen Monitor, a custom-made tetraphenylphosphonium (TPP⁺)-sensitive minielectrode, and a KCl bridge to an Ag/AgCl reference electrode connected to a pH meter. All instruments were connected to the data acquisition system. The incubation medium contained 125 mM KCl, 10 mM MOPS, pH 7.2, 2 mM MgCl₂, 2 mM KH₂PO₄, 10 mM NaCl, 1 mM EGTA, and 0.7 mM CaCl₂. At a Ca²⁺/EGTA ratio of 0.7, the free [Ca²⁺] is close to 1 μM, as determined using Fura-2. The substrate concentrations were as follows: 5 mM succinate without rotenone, 5 mM glutamate, 2.5 mM pyruvate, 10 mM α-ketoglutarate, and 2 mM malate. Oxidative phosphorylation (state 3) was initiated by the addition of 150 μM ADP. The uncoupled respiration (state 3U) was stimulated by titration with CCCP (0.05 μM aliquots) until the maximum rate of oxygen consumption was obtained.

**Measurements of Hydrogen Peroxide Generation**—H₂O₂ was measured using the Amplex red (Molecular Probes) method as described in Refs. 10 and 38. Respiratory inhibitors and uncou-
Glutamate Control of ATP and ROS Production

The fact that pyruvate and glutamate prevented or abolished inhibition of succinate oxidation suggests that the inhibition was associated with OAA. Because BM isolated in the absence of BSA (non-BSA-BM) showed inhibition of succinate dehydrogenase (Complex II) with practically normal rates of glutamate + malate oxidation (35), we analyzed interactions between substrates using non-BSA-BM.

Effects of Substrate Mixtures on Respiratory Activity of BM—Fig. 2 shows respiratory activities with the non-BSA-BM exposed to various combinations of respiratory substrates. The results were normalized to the rates seen with pyruvate + malate, which was taken as 100%. Fig. 2A shows that the resting respiration rates (state 4) with glutamate + malate and pyruvate + glutamate + malate were the same as with pyruvate + malate. With succinate, succinate + pyruvate, and succinate + glutamate, the state 4 respiratory rates were correspondingly 159% ($p < 0.001$), 146% ($p < 0.001$), and 135% ($p < 0.05$). When malate was also present, the rate of state 4 declined somewhat, and with succinate alone, malate inhibited the state 4 respiration by 43% ($p < 0.001$) (Fig. 2A). These relatively small effects of malate on the state 4 oxidation of succinate in the presence of pyruvate or glutamate result in significant inhibition of ROS generation, as shown in Figs. 5 and 6.

When BM oxidized pyruvate + glutamate + malate, the rate of oxidative phosphorylation (state 3) increased by 52% ($p < 0.001$) (Fig. 2B). With succinate alone, the state 3 respiration was only 44% of the control rate with pyruvate + malate ($p < 0.001$). With succinate + pyruvate or succinate + pyruvate + malate, the state 3 respiration rates increased to 115 and 106%, respectively (Fig. 2B). The increases were not statistically significant when compared with pyruvate + malate but were significantly higher when compared with succinate alone ($p < 0.001$; not shown). Glutamate or glutamate + malate added to BM oxidizing succinate also increased the state 3 respiration to the level observed with pyruvate + malate.

In BM uncoupled with CCCP, O$_2$ utilization in the presence of pyruvate + glutamate + malate was 71% ($p < 0.001$) higher than with pyruvate + malate (Fig. 2C). The initial rate of succinate oxidation by uncoupled BM was inhibited by 61% ($p < 0.001$), and the inhibition rapidly increased. It is likely that the inhibition was caused by OAA, which is consistent with the ability of pyruvate and glutamate to release the inhibition of succinate oxidation (Fig. 2C). With succinate alone, malate slightly inhibited the state 3 U respiration (Fig. 2C).

Response of Rat Heart Mitochondria to Substrate Mixtures—To study whether the effects of the substrate mixtures on respiratory activities, shown in Fig. 2, are specific for the BM only, we conducted similar experiments with mitochondria from rat heart isolated in the absence of BSA. Fig. 3 shows the rates of

![Figure 1](image-url)

**FIGURE 1.** Spontaneous inhibition of succinate oxidation by BSA-brain mitochondria and its release by glutamate and pyruvate. A, glutamate (G) (5 mM) and malate (M) (2 mM) were added to BM oxidizing succinate (5 mM) in the course of the experiment. B, succinate, glutamate, and malate were present before the addition of BM. The volume was 0.56 ml. 0.3 mg/ml BM was used in these experiments. Additions were as follows: TPP$^+$, added in 0.5 $\mu$M aliquots to a final concentration of 1.5 $\mu$M; ADP, 150 $\mu$M; CCCP, 0.6 $\mu$M. Numbers associated with the respiration traces are respiratory rates in ng atom of oxygen/min/mg of BM protein.

pler were added to the incubation media before the addition of mitochondria. The additions of resorufin or standard solutions of H$_2$O$_2$ (Fluka) were used for calibration of the fluorescence scale. Fluorimetric measurements were made using a highly sensitive fluorometer from C&L Co. (Middletown, PA).

**Data Acquisition**—Data acquisition was performed using hardware and software from C&L Co.

**Chemicals**—Sucrose, mannitol, and other chemicals were from Sigma and of molecular biology grade. All solutions were made using glass bidistilled water.

**Statistics**—Comparisons between two groups were made by unpaired t-test, and comparisons between more than two groups were made by analyses of variance followed by post hoc tests.

**RESULTS**

**Spontaneous Inhibition of Succinate Oxidation**—Nonsynaptic BM are commonly isolated with defatted BSA present either in the isolation medium (8, 10) or added to the mitochondrial suspension at some stage of the isolation procedure (37). It is believed that BSA binds fatty acids and thus prevents their uncoupling effect on BM (39).

An important feature of BM from Taconic Sprague-Dawley rats isolated with BSA was that 1–2 h postisolation, BM oxidizing succinate stopped responding to the addition of ADP or the uncoupler CCCP. Simultaneous measurements of respiration and membrane potential ($\Delta\psi$) in BM oxidizing succinate showed that the mitochondria remained depolarized after the initiation of ADP phosphorylation (Fig. 1A). When added to BM with inhibited succinate oxidation, glutamate or pyruvate restored normal response to ADP (Fig. 1A). In contrast, when succinate + glutamate (or pyruvate) were present from the beginning, the state 3 respiration and $\Delta\psi$ were not impaired (Fig. 1B). At the same time, oxidation of glutamate + malate or pyruvate + malate by the BSA-BM remained stable even 4–5 h after isolation with high respiratory control ratios (data not shown).
respiration in different metabolic states of the rat heart mitochondria (RHM). Unlike BM, the rates of oxygen consumption in the presence of pyruvate/glutamate/malate did not increase in RHM. Oxidation of succinate was significantly lower ($p < 0.01$) as compared with pyruvate/malate. In contrast to BM, with RHM, the rates of succinate oxidation did not depend on whether the mitochondria were isolated in the presence or absence of BSA. Pyruvate and glutamate added separately (data not shown) or together (Fig. 3, B and C) to the RHM oxidizing succinate increased respiration in metabolic states 3 and 3U to levels associated with pyruvate + malate, and the resting respiration (state 4) increased to a level significantly higher than that seen with pyruvate + malate ($p < 0.01$)

Effects of Substrate Mixtures on ROS Production by BM—We studied the effects of substrate mixtures on ROS generation by the non-BSA-BM (Fig. 4A) or BSA-BM (Fig. 4B) supported by glutamate and pyruvate. Fig. 7 summarizes the results of four separate experiments normalized for ROS generation with pyruvate + malate. Figs. 4A and 7 show that with glutamate + malate, generation of ROS was 4.4-fold lower than with pyruvate + malate and 9-fold lower than with pyruvate/glutamate/malate. When non-BSA-BM simultaneously oxidized pyruvate + glutamate + malate, the rate of ROS generation increased more than 2-fold when compared with pyruvate + malate (Fig. 4A). To analyze whether this increase in ROS production was associated with increased RET, we added malonate, an inhibitor of succinate dehydrogenase (SDH). Fig. 4A shows that the addition of 5 mM malonate inhibited ROS production with pyruvate + glutamate + malate by 77%. Malonate also inhibited ROS generation with pyruvate + malate by 50% (not shown) but had no effect on ROS generation with glutamate + malate or 10 mM α-ketoglutarate (α-KG) (data not shown). CCCP had effects similar to those of malonate on ROS.
generation with pyruvate + malate or pyruvate + glutamate + malate (data not shown). Fig. 4B shows that with the BSA-BM, ROS generation rates were higher than with the non-BSA-BM, but the pattern of the changes seen with the substrate mixtures described above were the same as those seen with the non-BSA-BM.

With succinate alone (Fig. 5), the rate of ROS generation by the BSA-BM was 12-fold higher when compared with the non-BSA-BM. With the BSA-BM, the addition of pyruvate or glutamate to the succinate-oxidizing BM had no effect or caused some inhibition of ROS generation, as compared with succinate alone (data not shown). With the non-BSA-BM, the rate of ROS generation supported by succinate + pyruvate + glutamate + malate was 4-fold higher than with succinate alone (Fig. 5). The addition of malonate or CCCP to the BSA-BM or non-BSA-BM oxidizing succinate alone completely inhibited ROS generation but caused only a partial inhibition with succinate + pyruvate + glutamate + malate with the non-BSA-BM (Fig. 5). Thus, both malonate and CCCP essentially eliminated only the succinate-supported portion of ROS production that depends on the energy-dependent RET.

Fig. 6 compares the effects of glutamate, pyruvate, and malate on oxidative phosphorylation (state 3) and ROS generation in metabolic state 4 normalized for BM oxidizing succinate alone, taken as 100%. During ATP synthesis, mitochondria do not produce ROS because of decreased membrane potential. In state 4, BM are fully energized and thus generate ROS. Fig. 6 shows that the addition of glutamate or pyruvate increased ATP production 2-fold and ROS production 1.5-fold. When glutamate and pyruvate were added together to the BM oxidizing the substrate mixtures described above, ATP production increased 2.5-fold, but ROS generation increased 4-fold. However, if malate was also added to the BM oxidizing the substrate mixtures described above, ATP production remained high, but ROS generation declined by 70–80%. Thus, malate can attenuate ROS generation without decreasing ATP production. Fig. 6 represents a typical experiment performed on the same batch of mitochondria. Fig. 7 shows the effects of substrate mixtures on ROS generation summarizing the results of four different experiments.

Effects of Substrate Mixtures on ROS Production by RHM—We studied the effects of the substrate mixtures on ROS generation using non-BSA RHMs. In accordance with the fact that
RHM did not increase respiration with glutamate/pyruvate/malate, no change in the rate of ROS production as compared with RHM oxidizing pyruvate/malate was observed (Fig. 8A). Likewise, the addition of malonate had no effect on ROS generation by RHM (Fig. 8A).

Fig. 8B shows that with succinate as a substrate, the addition of glutamate + pyruvate to non-BSA-RHM caused a significant increase in ROS production ($p < 0.01$). However, when malonate was also added, generation of ROS was inhibited by 50% and was 38% lower than with succinate alone (Fig. 8B).

**DISCUSSION**

It is now a well established fact that an appreciable amount of the neuromediators glutamate and y-aminobutyric acid are transported from synaptic clefts into the postsynaptic elements of neurons (20, 27–29). A large scale utilization by BM of glutamate as the energy-delivering substrate is strongly suggested by the high number of synapses found in the brain, which mostly release glutamate in the synaptic clefts (32, 33). In BM, there are intimate relationships between the tricarboxylic acid cycle metabolites and amino acids (40). BM possess three enzymes (alanine aminotransferase, aspartate aminotransferase, and glutamate dehydrogenase) that convert glutamate to $\alpha$-KG (41, 42). The enzymes are frequently found colocalized, and they act in a concerted manner (41). The three glutamate-transforming enzymes have high Michaelis constants for glutamate: for glutamate dehydrogenase, 8–17 mM (43); for alanine aminotransferase and aspartate aminotransferase, 7–20 mM (42). However, in view of the electrogenic nature of the AGC and removal of $\alpha$-KG by the tricarboxylic acid cycle (14), in the energized BM, the three glutamate-transforming reac-

**FIGURE 4.** Stimulation by glutamate of H$_2$O$_2$ generation by brain mitochondria oxidizing pyruvate and malate does not depend on whether BM were isolated in the absence (non-BSA-BM) or presence of BSA (BSA-BM). A, non-BSA-BM; B, BSA-BM (see “Experimental Procedures” for details). The incubation conditions were as in Fig. 1. Final volume was 1 ml. Additions were as follows: 5 $\mu$M Amplex red, 3 units of horseradish peroxidase, 50 units of superoxide dismutase (Sigma), 0.1 mg of rat brain mitochondria, 5 mM glutamate (G) + 2 mM malate (M), 2.5 mM pyruvate (P) + 2 mM malate. Malonate 5 mM was added before BM. Numbers associated with the traces show the rates of H$_2$O$_2$ production in pmol of H$_2$O$_2$/min/mg of BM protein. AU, arbitrary units.

**FIGURE 5.** Glutamate and pyruvate stimulate the succinate supported H$_2$O$_2$ generation only in the non-BSA-brain mitochondria. Incubation conditions were as in Fig. 1. Final volume was 1 ml. Additions were as follows: 5 $\mu$M Amplex red, 3 units of horseradish peroxidase, 50 units of superoxide dismutase (Sigma), 0.1 mg of rat brain mitochondria, 5 mM succinate (S), 5 mM glutamate (G), 2.5 mM pyruvate (P), 2 mM malate (M). Malonate (5 mM) was added before BM. Numbers associated with traces show the rates of H$_2$O$_2$ production in pmol of H$_2$O$_2$/min/mg of BM protein. AU, arbitrary units.

**FIGURE 6.** A comparison of the effects of substrate mixtures on the succinate supported state 3 respiration and state 4 generation of H$_2$O$_2$ by non-BSA-brain mitochondria. Incubation conditions were as in Figs. 4 and 5. All experiments were made on the same batch of mitochondria. Data were normalized to the succinate alone, taken as 100%. S, succinate (5 mM); P, pyruvate (2.5 mM); G, glutamate (5 mM); M, malate (2 mM).
tions operate irreversibly with the net loss of glutamate. Therefore, even at concentrations much lower than the $K_m$ values of the enzymes, glutamate will be rapidly oxidized.

Activation of Oxidative Phosphorylation during Simultaneous Oxidation of Pyruvate, Glutamate, and Malate—During neuronal activation, astrocytes provide additional amounts of lactate for postsynaptic neuronal mitochondria (4, 13). For the rapid conversion of lactate to pyruvate, postsynaptic mitochondria must receive a certain amount of glutamate to fuel MAS in order to recycle cytosolic NAD$^+$ (14). It is important to note that the activity of the brain mitochondrial isoform of the AGC is controlled by Ca$^{2+}$ (17). Therefore, increased neuronal activity activates MAS via small changes in the extramitochondrial Ca$^{2+}$, whereas increased mitochondrial Ca$^{2+}$ activates mitochondrial respiration (17).

Our data obtained with purified nonsynaptic mitochondria, which originate predominantly from postsynaptic elements of the synapses, neuronal cell bodies, and astroglia (14, 32), further demonstrate the advantages of simultaneous fueling of BM with glutamate + pyruvate for increased energy demands in activated neurons. Fig. 2, B and C, shows that when BM oxidized pyruvate + glutamate + malate, the rates of respiration increased by 52% in state 3 and 71% in state 3U, as compared with pyruvate + malate. This shows that when present together, pyruvate + glutamate, particularly when malate was also present, overcome some limiting steps in the hydrogen supply. Here we consider two such limiting steps.

First, the activity of SDH/Complex II is inhibited by OAA, thereby limiting the rate of the tricarboxylic acid cycle operation during state 3 and state 3U. The ability of pyruvate and glutamate to overcome inhibition of SDH (see Figs. 1A and 2, B and C) is probably associated with the removal of OAA in the citrate synthase and aspartate aminotransferase reactions, respectively.

The second limiting step is the $\alpha$-ketoglutarate dehydrogenase complex ($\alpha$-KGDHC) reaction. It has been shown that the activity of $\alpha$-KGDHC is the lowest among the tricarboxylic acid cycle enzymes (44) and is controlled by availability of $\alpha$-KG (45) and the enzyme’s affinity for $\alpha$-KG, which is controlled by Ca$^{2+}$ and Mg$^{2+}$ (46, 47). In addition, a decreased matrix ATP/ADP ratio due to increased energy consumption in activated neurons would also increase the availability of GDP for the substrate level phosphorylation and thus the overall activity of $\alpha$-KGDHC (41). The key role of $\alpha$-KG for the tricarboxylic acid-related hydrogen supply was demonstrated in experiments with labeled metabolites (48). Balazs (48) has shown that in BM oxidizing glutamate + pyruvate + malate, the amount of $\alpha$-KG increased 30 times. Under these conditions, glutamate dehydrogenase does not participate in production of $\alpha$-KG (49).

Since aspartate aminotransferase and alanine aminotransferase are present in great excess, compared with the respiration rate, the OAA formed is continuously removed by the transamination reactions. Balazs (49) concluded that competition between the $\alpha$-KGDHC and glutamate dehydrogenase occurs, probably for NAD$^+$, resulting in preferential oxidation of $\alpha$-KG.
There is evidence (18) that in the presence of glutamate + pyruvate, the tricarboxylic acid cycle in brain mitochondria operates as two coupled cycles; one (cycle A) leads from pyruvate to OAA, and another (cycle B) leads from OAA to α-KG, which includes the citrate synthase reaction (see Fig. 9). According to Yudkoff et al. (18), the flux of substrates through cycle A is 3–5-fold faster than through cycle B. Thus, with pyruvate + glutamate + malate, activation of α-KGDHC and SDH may significantly increase the rates of the tricarboxylic acid cycle and respiratory chain in state 3 and state 3U (Fig. 2). A high turnover of cycle A with activated SDH would increase RET and the associated ROS production.

Our experiments with the RHM suggest that activation of oxidative phosphorylation and increased ROS generation with pyruvate + glutamate + malate are specific to BM. More recently, we have seen the same result with spinal cord mitochondria (36). However, activation of succinate oxidation by pyruvate and glutamate may be a common phenomenon for mitochondria from many organs.

**Stimulation of Succinate Oxidation by Pyruvate or Glutamate**—OAA is the most powerful inhibitor of SDH (50). The affinity of SDH for OAA changes with the reduction of the enzyme’s sulfhydryl group depending on mitochondrial energization (51). Upon de-energization of mitochondria, the affinity of SDH to OAA increases 10-fold (51). Another important property of SDH is that, besides succinate, the enzyme can also oxidize malate with similar affinity (52). Although externally added OAA competes with succinate for binding to the enzyme, OAA formed during oxidation of malate remains tightly bound to the enzyme (52). Therefore, malate is a powerful inhibitor of SDH (52).

**ROS Production by Neuronal Mitochondria Oxidizing Substrate Mixtures**—Fueling of the BM with pyruvate + malate or pyruvate + glutamate + malate led to a 4- and 9-fold increase, respectively, in the state 4 ROS generation, as compared with glutamate + malate (Fig. 4). Figs. 4, A and B, and 5 show that malonate, a potent blocker of SDH, only partially inhibited ROS production supported by pyruvate + malate or pyruvate + glutamate + malate. CCCP had effects similar to those of malonate (not shown). Malonate had no effect on ROS production with glutamate + malate or 10 mM α-KG + malate (data not shown).

At the same time, malonate and CCCP completely inhibited ROS generation with succinate alone (Fig. 5B). These results suggest that during oxidation of pyruvate + malate or pyruvate + glutamate + malate, up to 50–70% of the ROS generation was associated with increased production of succinate in the tricarboxylic acid cycle and the resulting RET. Importantly, increased ATP production (in state 3) and increased ROS generation (in state 4) with glutamate + pyruvate + malate were seen only with BM and spinal cord mitochondria (36) and were observed with both non-BSA and BSA-BM.

Fig. 5 shows that with the BSA-BM, the succinate-supported ROS generation was 12 times higher than with the non-BSA-BM. We suggest that the dramatic increase in ROS generation in BSA-BM may be explained in the following way. It is known that BSA has a relatively high affinity for binding organic anions. Therefore, in the presence of the high capacity phase of the isolation medium containing BSA, which exceeds the mitochondrial phase by several orders of magnitude, OAA may be removed from the catalytic center of SDH. We also found that BSA has a rather strong antioxidative effect. Therefore, BSA may protect BM from oxidative damage during isolation and thus increase ROS production because of better energization of the mitochondria. The stimulatory effects of glutamate and pyruvate on the succinate-supported ROS production were observed only with the non-BSA-BM (Figs. 5 and 6). Similar results were published recently by Zoccarato et al. (53), who incubated BM in the presence of 0.5 mg/ml of defatted BSA. Muller et al. (54) also found that glutamate had no stimulatory effect on the succinate-supported ROS generation with skeletal muscle mitochondria isolated in the presence of BSA. Our experiments with the RHM oxidizing succinate showed that both respiration and ROS generation did not depend on the presence of BSA in the isolation medium (data for the BSA-RHM are not shown), and the addition of glutamate + pyruvate + malate significantly stimulated respiration at all metabolic states ($p < 0.001$) (Fig. 3). Thus, stimulation of the succinate-driven ROS generation by glutamate or pyruvate is not specific only for BM and spinal cord mitochondria.

We suggest that inhibition of SDH by OAA may represent a universal defensive mechanism to minimize ROS generation in resting mitochondria. In this respect, malate may be regarded as a metabolite that can control ROS generation in vivo through regulation of the activity of the succinate-driven RET. As shown in Fig. 6, malate had little influence on the rate of ATP synthesis supported by succinate + glutamate but strongly inhibited the state 4 generation of ROS in BM (Figs. 5 and 6) and RHM (Fig. 8B) (see also Refs. 35, 36, and 54).

Based on the data presented, we put forward the following hypotheses. (i) During neuronal activity, the postsynaptic mitochondria are exposed to increased levels of pyruvate and glutamate, which enhance ATP production due to specific interactions between the glutamate-transforming enzymes and the tricarboxylic acid cycle enzymes. This effect of the substrate mixtures is specific for BM and spinal cord mitochondria (36). As soon as activation of a synapse is terminated, transport of glutamate from the synaptic cleft would also stop. As a result, activation of respiration induced by the simultaneous presence
of glutamate and pyruvate would also be halted because glutamate becomes exhausted. Thus, the neuromediator glutamate controls energy metabolism in the brain not only at the level of astrocytes by enhancing production of lactate but also at the level of neurons by controlling the activity of MAS and specific interactions between aminotransferases. (ii) In the absence of neuronal activation, SDH (Complex II) in the nonsynaptic mitochondria is inhibited by OAA. The physiological significance of this inhibition is to prevent excessive ROS production associated with reverse electron transport. This is important, because with pyruvate, which is the major mitochondrial substrate in resting neurons (12, 17), there is a substantial production of succinate and thus a possibility of increased RET-dependent ROS production (see Fig. 4, A and B). During increased neuronal activity, glutamate and pyruvate temporarily release the inhibition of SDH. However, increased ATP production (state 3) prevents the generation of ROS. Kudin et al. (8), using BSA-BM, estimated that up to 1% of oxygen may be converted to superoxide. Our data show that with the non–BSA mitochondria, the rate of ROS production was more than 10 times lower (in vivo) than with BSA-BM, estimated that up to 1% of oxygen may be converted to superoxide. Thus, according to our hypothesis, in resting neurons in vivo, only 0.1–0.2% of oxygen consumed may be converted to superoxide. Because the intrinsic inhibition of SDH (Complex II) is not brain-specific, it may represent a universal mechanism of protection against oxidative stress associated with reverse electron transport.

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