Methylene Blue and Epinephrine: A Synergetic Association for Anaphylactic Shock Treatment*

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Background: Severe hypotension resulting from anaphylactic shock may be refractory to epinephrine and impair cerebral oxygenation and metabolism contributing to anaphylactic shock morbidity and mortality. Refractoriness to epinephrine could be corrected by nitric oxide pathway inhibitors such as methylene blue.

Objectives: To compare the systemic and regional (brain and skeletal muscle) effects of epinephrine and methylene blue given alone or in combination in a rat model of anaphylactic shock.

Design: Prospective laboratory study.

Setting: University laboratory.

Subjects: Male Brown-Norway rats ($n = 60$).

Interventions: After sensitization and induction of anaphylactic shock by ovalbumin, animals received either vehicle (ovalbumin group) or a 3-mg/kg methylene blue bolus (methylene blue group) or epinephrine (epinephrine group) or both (methylene blue–epinephrine group). Sensitized control rats received only vehicle and no ovalbumin (control group).

Measurement and Main Results: Mean arterial pressure, cardiac output, cerebral blood flow, skeletal muscular oxygen partial pressure, cerebral oxygen partial pressure, skeletal muscular, and cerebral interstitial lactate/pyruvate ratio were measured. Cleaved caspase 3 and hypoxia-inducible factor-1α expression were analyzed in the cerebral cortex by Western blot. Without treatment, rats died rapidly within 15 mins from a decrease in cardiac output and mean arterial pressure, whereas treated rats survived until the end of the experiment. Methylene blue alone extended survival time but without significant improvement of hemodynamic variables and tissue perfusion and did not prevent neuronal injury. Epinephrine restored partially systemic hemodynamic variables and cerebral perfusion preventing glutamate-induced excitotoxicity. Compared with epinephrine alone, the methylene blue–epinephrine association avoided neuronal excitotoxicity and had an additive effect both on hemodynamic variables and for prevention of brain ischemia. Neither treatment could significantly restore cardiac output or prevent muscular compartment ischemia and microvascular leakage.

Conclusions: Anaphylactic shock is associated with severe impairment of cerebral blood flow despite correction of arterial hypotension. Epinephrine must still be considered as the first-line vasoconstrictive agent to treat anaphylactic shock. The epinephrine–methylene blue association was the most effective treatment to prevent cerebral ischemia and could be used in anaphylactic shock refractory to epinephrine. (*Crit Care Med* 2013; 41:195–204)

Key Words: anaphylaxis; cerebrovascular circulation; epinephrine; ischemia; methylene blue; microdialysis; nitric oxide; shock

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Anaphylactic shock (AS) is a relatively common problem in the perioperative period with a prevalence of one to nine per 10,000 general anesthetic procedures worldwide (1). AS is an acute, potentially fatal reaction within the spectrum of generalized immediate-type hypersensitivity (i.e., anaphylaxis). During anesthesia, this shock is lethal in 3% to 10% of patients (2, 3) or can lead to severe morbidity (cerebral anoxia). Epinephrine (EPI), recommended by guidelines, may fail to restore rapidly adequate organ perfusion or can be completely ineffective; in this case, the shock is considered refractory to catecholamines for reasons that are not understood (4, 5).

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AS is commonly classified as a distributive shock characterized by hypovolemia resulting from increased capillary permeability and arterial hypotension resulting from excessive vasodilatation. However, recent reports indicate that the pathophysiology of anaphylaxis is more complex than initially thought.

Impaired myocardial contractility, an increase in venous tone in the splanchnic and portal vascular beds or in the pulmonary veins, and a severe decrease in blood perfusion in skeletal muscles have been demonstrated (6–10). On the contrary, the consequences on cerebral vasculature and brain perfusion remain to be determined, whereas these changes may be of critical importance in delineating optimal therapeutic goals and strategies (11).

The mentioned effects of AS on organ function are related to the explosive release of mediators such as histamine, platelet activating factor, leukotrienes, and tumor necrosis factor-α (12, 13) leading to the activation of multiple pathways that include but are not limited to the nitric oxide (NO) pathway (14). Despite the documented myocardial depressive effect of NO (15) and the potential beneficial effect related to its inhibition, the effect of pharmacological inhibition of the NO pathway on early survival in AS remains controversial, depending on experimental models, animal species, and NO pathway inhibitors used (16–18). It is possible that inhibition of the NO pathway could have beneficial effects on one organ (e.g., the heart) but be deleterious on other organs (e.g., the brain).

Furthermore, the interactions between EPI and the different drugs known to pharmacologically inhibit the NO pathway have not been thoroughly investigated. Therefore, the purpose of our study was to investigate the beneficial or detrimental role of methylene blue (MB, a substance known to inhibit one of the final effectors of NO, i.e., guanylyl cyclase) alone or in association with EPI, on early survival, hemodynamic, tissue oxygen availability, and metabolic disturbances, with special emphasis on brain consequences, in an anesthetized rat model of Ig-E–mediated AS.

Because long-term consequences of treatments cannot be investigated because of the surgical procedure required in our experiments, brain-cleaved caspase 3 and hypoxia-inducible factor (HIF)-1α expressions were analyzed as surrogate markers of AS-induced delayed injury in the cerebral cortex.

MATERIALS AND METHODS

Animals and Sensitization Protocol

After approval by our institutional Animal Care Committee, all animal procedures and care were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC). The animals had free access to water and food.

Ten-week-old Brown-Norway rats weighting 250 g–300 g (Janvier, Le Genest-St-Isle, France) were used for these experiments. They were kept under standard conditions (temperature 21 ± 1°C; light from 6 AM to 6 PM) and given a standardized diet (A04; UAP, Villemoisson-sur-Orge, France) and water (Aqua-clear; Culligan, Northbrook, IL) ad libitum. On days 0, 4, and 14, rats were sensitized by subcutaneous administration of 1 mg grade VI chicken egg albumin (ovalbumin [OVA]; Sigma-Aldrich, Saint-Quentin Fallavier, France) and 4 mg aluminum hydroxide in adjuvant (Sigma, St. Louis, MO) diluted in 1 mL 0.9% saline solution, as previously described (7).

Surgical Procedure, Measurement of Hemodynamic Variables, Tissue Oxygen Partial Pressure, and Interstitial Microdialysis

The surgical procedure was performed on day 21 after the initial sensitization. Anesthesia was induced with 3% isoflurane and maintained with 1% isoflurane. The trachea was cannulated, and the lungs were mechanically ventilated with 100% oxygen using a Harvard rodent respirator (Model 683; Harvard Apparatus, Cambridge, MA). Tidal volume was 2.5 mL, and respiratory rate was adjusted to maintain $PaCO_2$ between 38 mm Hg and 42 mm Hg. Rectal temperature was maintained by an intermittent heating light at 38 ± 0.5°C throughout the experiment. A fluid-filled polyethylene catheter (inside diameter, 0.58 mm; outside diameter, 0.96 mm; Biotrol Diagnostic, Chennevières Les Louvres, France) was inserted in the left femoral artery for pressure monitoring. Mean arterial pressure (MAP) and heart rate were recorded continuously using a strain gauge pressure transducer (DA-100; Biopac Systems, Northborough, MA). Another fluid-filled catheter was inserted in the left femoral vein for administration of drugs and a basal physiological need perfusion of 0.9% saline solution at 10 mL/kg·hr. Skeletal muscle and cerebral metabolisms were studied in two independent experimental series.

Specific Skeletal Muscle Procedure

A thermistor catheter connected to a thermodilution cardiac output (CO) computer (Cardiomax III, Columbus Instrument, Columbus, OH) was inserted in the left carotid artery and moved into the left ventricle for CO monitoring.

A flexible Clark-type polarographic oxygen electrode (diameter, 500 μm; length, 200 mm) computer-supported LICOX system (GMS, Mielkendorf, Germany) was introduced in the right quadriceps muscle for tissue oxygen partial pressure measurement ($mPtiO_2$).

In the left quadriceps muscle, a liner flexible microdialysis probe (polyarylethersulfone membrane; membrane length, 10 mm; outside diameter, 0.5 mm; molecular weight cutoff, 20 kd; CMA/Microdialysis AB, Kista, Sweden) was inserted as previously described (7). The probe was connected to a 2.5-mL CMA/106 microsyringe mounted on a microinjection pump (CMA 107 Microdialysis Pump, Stockholm, Sweden) and infused at ambient temperature with a lactate-free Ringer's solution (Na^+ 147 mmol/L, K^+ 4 mmol/L, Ca^{2+} 2.3 mmol/L, Cl^- 156 mmol/L) at a flow rate of 2 μL/min. A period of 30 mins was allowed for equilibration before beginning sample collection. Microdialysates were collected in microvials (CMA) every 10 mins until the end of the experiment, starting 10 mins before shock induction. Samples were kept on ice initially and then stored at –20°C. The interstitial lactate, glucose, pyruvate,

and glutamate were measured with the CMA 600 microdialysis analyzer.

Specific Cerebral Procedure

Cerebral $PtiO_2$ ($cPtiO_2$) was monitored by a flexible Clark-type polarographic oxygen electrode (outside diameter, 0.5 mm, membrane length 200 mm, 4-mm sensitive area; Licox CC1.R, Integra NeuroSciences, Sophia Antipolis, France) connected to LICOX CMP instrument (Integra NeuroSciences) and inserted into the hippocampus (4.6 mm anterior and 2 mm lateral to the λ to a depth of 3 mm).

Cerebral blood flow (CBF) was monitored using a laser-Doppler needle probe (PERIFLUX systems, probe 411, diameter 450 μm) connected to a PeriFlux PF 5010 laser Doppler monitor (Perimed AB, Stockholm, Sweden) inserted into the right cerebral cortex 2 mm anterior to the oxygen electrode.

A central nervous system-designed microdialysis probe (CMA/12, polyarylethersulfone membrane, length 3 mm, outside diameter, 0.5 mm, cutoff 20 kd, CMA/Microdialysis AB) was implanted through a hole placed 7.3 mm anterior and 4 mm lateral to the λ in the striatum to a depth of 6 mm, and probes were infused with CMA isotonic sterile perfusion fluid (NaCl 147; KCl 2.7; $CaCl_2$ 1.2; $MgCl_2$ 0.85 mmol/L) at a flow rate of 2 μL/min.

Induction of Shock and Treatment With MB and/or EPI

After a 30-min stabilization period, AS was induced by intravenous injection of 1 mg ovalbumin (T0). Animals were randomly assigned to five different groups: vehicle in control (CON) group nonshocked, no-treatment in OVA group, MB only, EPI only, and MB–EPI. The investigator was not blinded to the drugs used. A single bolus of MB (Sigma) of 3 mg/kg was injected 3 mins after shock induction (T3). The first bolus of EPI of 2.5 μg was injected at T3 and a second bolus at T5, continuous infusion of EPI 10 μg/kg·min, was initiated immediately after the first bolus. In the group receiving the combined treatment, MB bolus was injected immediately after the first EPI bolus. MAP, heart rate, CO, CBF, $mPtiO_2$, and $cPtiO_2$ were recorded at the following time points: T0 (before shock induction), T1, T2.5, T5, T7.5, T10, T12.5, T15, T17.5, T20, and then every 5 mins until the end of experiment. Arterial blood samples were taken from each animal before the shock induction and at the end of the experiment for measurement of arterial blood gas and plasma sodium, chloride, lactate, nitrite, nitrate, and hemoglobin. Samples for plasma nitrite and nitrate analysis were stored at −80°C. They were then deproteinized before analysis using Vivaspin 500 centrifugal concentrators (Sigma-Aldrich, St. Louis, MO). Plasma concentrations of nitrite and nitrate were measured according to Griess reaction by enzyme-linked immunosorbent assay (Parameter colorimetric competitive enzyme-linked immunosorbent assay kit; R&D Systems, Abingdon, UK) according to the manufacturer's instructions. The animals were euthanized by an overdose of thiopentone sodium after the last blood sample collection.

Western Blot Analysis

Cerebral cortex tissues were homogenized with a Polytron homogenizer (Tissue-Lyser II; Qiagen, Courtaboeuf, France). Protein content was measured by the Bradford method (Pierce, ThermoScientific, Brebières, France). Protein extracts from cerebral cortex tissues (20 μg) were separated by a 4% to 12% Criterion XT Bis-Tris Gel (BioRad, Marnes-la-Coquette, France) and subjected to Western blotting for rabbit anti-HIF-1α (1:2000; Abcam, Paris, France) and rabbit anticleaved caspase 3 (1:1000; Cell Signaling, Ozyme, Saint-Quentin en Yvelines, France), and β-actin was used as a protein loading CON. Bound antibody density analyses were performed by LAS-4000 imager (FSVT, Courbevoie, France) and Multi-Gauge software (LifeScience, Fujifilm, France). After densitometry analyses, optical density values were expressed as arbitrary units (AUs).

Statistical Analysis

Results are expressed as mean ± SEM. Intragroup and among-group comparisons were performed using one-way and two-way analysis of variance for repeated measures. Differences

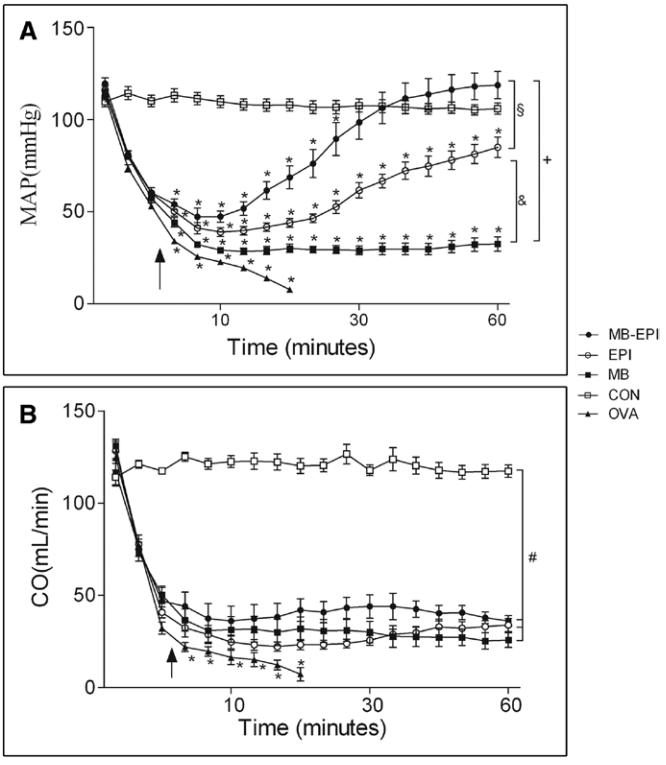

Figure 1. Time course of systemic mean arterial pressure (MAP) (**A**, $n = 12$ rats/group) and cardiac output (CO) (**B**, $n = 6$ rats/group) values in ovalbumin (OVA), control (CON), methylene blue (MB), epinephrine (EPI), and epinephrine associated with methylene blue (MB–EPI) groups. Time 0 corresponds to the injection of OVA. Values are presented as mean ± SEM. ↑ corresponds to the beginning of treatment. $^{*}p \leq 0.05$ vs. the CON group, $^{+}p \leq 0.05$ between-group differences MB–EPI group vs. MB group, $^{\&}p \leq 0.05$ between-group differences EPI group vs. MB group, $^{\S}p \leq 0.05$ between-group differences MB–EPI group vs. EPI group, $^{\#}p \leq 0.05$ between-group differences MB–EPI or EPI or MB groups vs. CON group. Triangle = OVA group; white square = CON group; black square = MB group; white circle = EPI group; black circle = MB-EPI group.

between groups at a time period were analyzed using the unpaired *t* test for nonrepeated measures with Statview 5.0 software (Deltasoft, Meylan, France). When a significant interaction was observed with two-way analysis of variance, paired comparisons were made with the Fisher's post hoc test.

RESULTS

Sixty OVA-sensitized Brown-Norway rats (274 ± 4 g) were studied and randomly allocated to the CON group (no-shock, $n = 12$), OVA group (no-treatment, $n = 12$), MB group ($n = 12$), EPI group ($n = 12$), and MB–EPI group ($n = 12$). Because animals from the OVA group died within 15 mins, data from microdialysis, Western blot, and plasma biochemistry were not obtained from this group.

Systemic Hemodynamic Variables

Time-course profiles for MAP (**Fig. 1*A***) and CO (Fig. 1*B*) were different among the groups. Hemodynamic variables remained relatively stable throughout the entire study period in CON animals, whereas OVA injection resulted in a similar rapid and profound decrease of MAP and CO in the other groups. Without treatment, rats died within15 mins with a dramatic decrease in CO and MAP, whereas treated rats survived until the end of the experiment. Treatment resulted in stabilization of MAP at a low value in the MB group. MAP increased progressively, reaching 70% of the basal value at the end of the experiment in the EPI group, and was completely restored as of T30 min in the MB–EPI group. The three treatments enhanced CO as compared with the OVA group; after the initial decrease, CO remained stable at low values in the MB group. EPI alone or in association with MB restored poorly CO at 26% and 31% of basal value, respectively but without a significant difference among these three groups.

Effects of the Different Treatments in the Cerebral Compartment

Cerebral hemodynamic and metabolic variables remained stable during the entire study period in the CON rats. In the absence of treatment, a rapid and profound decrease in CBF and $cPtiO_2$ was observed after shock induction (**Fig. 2A–*B***). A similar decrease in CBF was observed in the treated groups up to 12.5 mins after shock induction. Subsequently, CBF stabilization occurred at low values in the MB group from 106 ± 2 to 22 ± 3 PU, whereas EPI alone or in association with

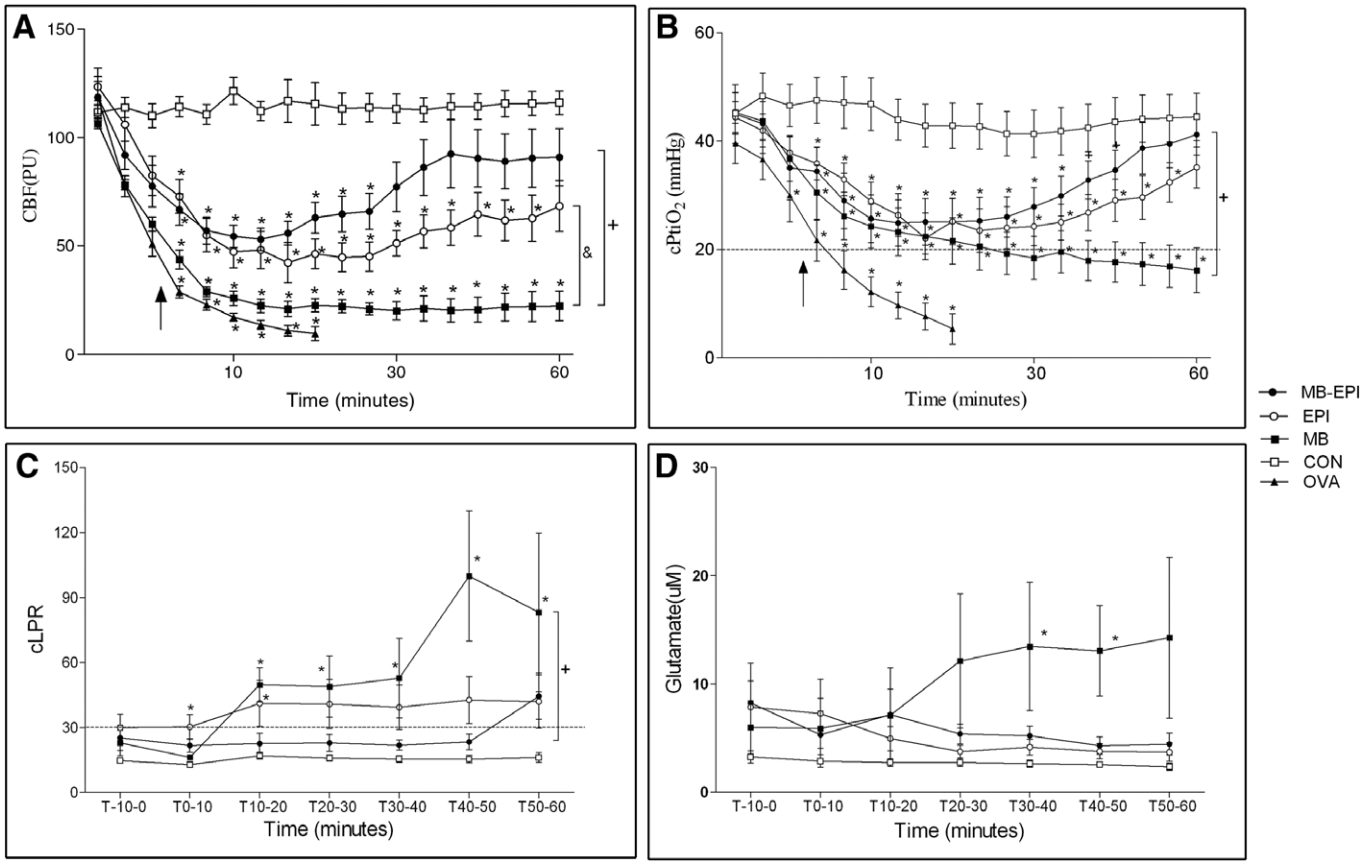

Figure 2. Time course of cerebral cortical blood flow (CBF) (**A**), tissue oxygen partial pressure ($PtiO_2$) in the hippocampus (**B**), lactate/pyruvate ratio (cLPR) in the striatum (**C**), and interstitial glutamate (**D**) in striatum values in ovalbumin (OVA), control (CON), methylene blue (MB), epinephrine (EPI), and epinephrine associated with methylene blue (MB–EPI) groups ($n = 6$ rats/group). Because of the microdialysis flow rate, samples were collected every 10 mins, starting 10 mins before shock induction (T0). OVA rats died within 15 mins, so their data were not measured. Values are presented as mean ± SEM. ↑ corresponds to injection of treatment; the *dotted line* corresponds to the ischemic threshold. $^{*}p \leq 0.05$ vs. the control group, $^{+}p \leq 0.05$ between-group differences MB–EPI group vs. MB group, $^{\&}p \leq 0.05$ between-group differences EPI group vs. MB group. Triangle = OVA group; white square = CON group; black square = MB group; white circle = EPI group; black circle = MB-EPI group.

TABLE 1. Time Course of Interstitial Lactate, Pyruvate, and Glucose Concentrations in the Brain ($n = 6$ rats/group)

Group		Control Group	Methylene Blue Group	Epinephrine Group	Epinephrine Associated With Methylene Blue Groups
Lactate (mM)	T10 to T0	0.32 ± 0.07	0.20 ± 0.04	0.34 ± 0.14	0.27 ± 0.04
	T0 to T10	0.23 ± 0.05	0.23 ± 0.04	0.32 ± 0.08	0.23 ± 0.01
	T10 to T20	0.30 ± 0.05	0.59 ± 0.11[a]	0.40 ± 0.02	0.43 ± 0.06
	T20 to T30	0.25 ± 0.02	0.55 ± 0.12[a]	0.40 ± 0.04	0.47 ± 0.10
	T30 to T40	0.24 ± 0.02	0.50 ± 0.13[a]	0.36 ± 0.04	0.33 ± 0.08
	T40 to T50	0.24 ± 0.03	0.50 ± 0.14	0.31 ± 0.01	0.36 ± 0.08
	T50 to T60	0.24 ± 0.03	0.39 ± 0.12	0.27 ± 0.03	0.31 ± 0.06
Pyruvate (mM)	T10 to T0	0.020 ± 0.003	0.012 ± 0.004	0.011 ± 0.003	0.015 ± 0.004
	T0 to T10	0.017 ± 0.002	0.015 ± 0.003	0.011 ± 0.002	0.012 ± 0.002
	T10 to T20	0.017 ± 0.001	0.014 ± 0.004	0.012 ± 0.003	0.023 ± 0.006
	T20 to T30	0.016 ± 0.002	0.014 ± 0.003	0.011 ± 0.003	0.022 ± 0.005
	T30 to T40	0.016 ± 0.002	0.014 ± 0.003	0.012 ± 0.003	0.016 ± 0.004
	T40 to T50	0.017 ± 0.001	0.007 ± 0.002[a]	0.010 ± 0.003	0.017 ± 0.005[b]
	T50 to T60	0.016 ± 0.002	0.008 ± 0.003	0.010 ± 0.003	0.010 ± 0.003
Glucose (mM)	T10 to T0	0.22 ± 0.03	0.21 ± 0.06	0.23 ± 0.07	0.26 ± 0.05
	T0 to T10	0.25 ± 0.09	0.17 ± 0.05	0.26 ± 0.05	0.25 ± 0.03
	T10 to T20	0.35 ± 0.09	0.071 ± 0.03[a]	0.23 ± 0.01	0.28 ± 0.04[b]
	T20 to T30	0.23 ± 0.08	0.11 ± 0.05	0.23 ± 0.04	0.30 ± 0.05[b]
	T30 toT40	0.23 ± 0.05	0.11 ± 0.05	0.30 ± 0.03[b]	0.34 ± 0.05[b]
	T40 to T50	0.27 ± 0.04	0.13 ± 0.05	0.28 ± 0.04[b]	0.38 ± 0.06[b]
	T50 to T60	0.25 ± 0.03	0.11 ± 0.06	0.31 ± 0.03[b]	0.28 ± 0.07[b]

Time 0 (T0) corresponds to injection of 1 mg ovalbumin.
[a]$p \leq 0.05$ vs. the control group.
[b]$p \leq 0.05$ vs. the methylene blue group.

MB restored partially CBF (55% and 77% from basal values, respectively; Fig. 2*A*).

After shock induction, a significant decrease in $cPtiO_2$ was initially observed in each treatment group. Subsequently, in the MB group, $cPtiO_2$ decreased slightly with time, reaching values below the cerebral ischemic threshold ($cPtiO_2$ <20 mm Hg) as of T25 mins. These low $cPtiO_2$ values in the MB group, reflecting ischemia, were in agreement with the decrease of interstitial glucose concentrations (**Table 1**), the progressive increase in L/P ratio (Fig. 2*C*), and the release of glutamate (Fig. 2*D*).

In contrast, EPI alone or in association with MB restored progressively O_2 availability (79% and 92%, respectively, of basal values; Fig. 2*B*). This was confirmed by cerebral microdialysis results confirming a relative stability of glucose interstitial concentrations and L/P ratio (Fig. 2*C*) and the absence of increased glutamate release reflected by a moderate increase in interstitial glucose concentrations (Table 1) and glutamate decrease (Fig. 2*D*).

Effects of the Different Treatments in the Muscular Compartment

The time-course profiles of $mPtiO_2$ (**Fig. 3*A***) were similar in all treated groups. A rapid and sustained decrease in $mPtiO_2$ was observed after shock induction. The basal values were approximately 40 mm Hg, decreased rapidly after shock induction, and stabilized finally at low values without restoration. The effects of EPI alone, MB–EPI, and MB (6.8 ± 1.6 mm Hg, 6.8 ± 1.6 mm Hg, and 4.2 ± 2.0 mm Hg, respectively) on oxygen availability were not statistically different.

Interstitial muscular lactate concentrations increased progressively and interstitial pyruvate concentrations decreased progressively with time without differences among treatment groups (**Table 2**). A progressive increase in interstitial muscular

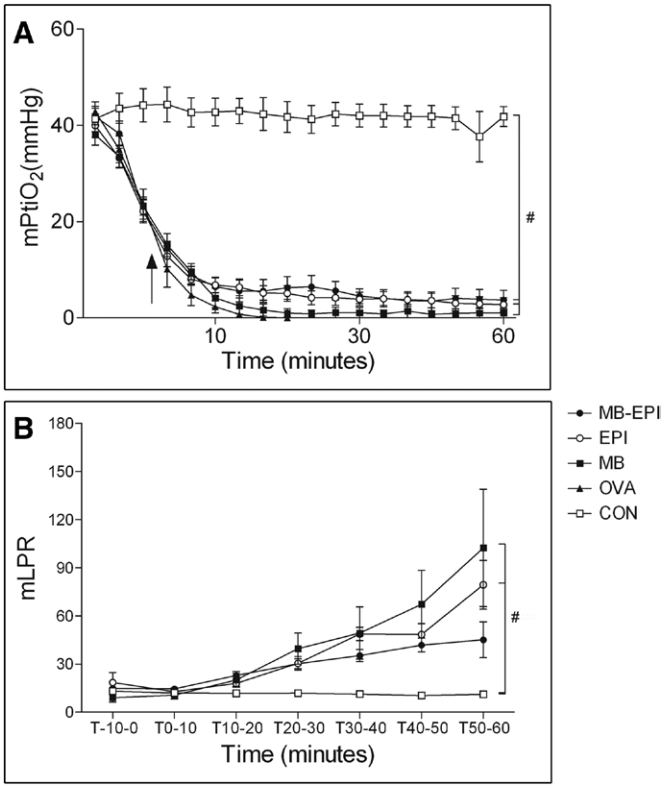

Figure 3. Time course of tissue oxygen partial pressure ($mPtiO_2$) (**A**) and lactate/pyruvate ratio (mLPR) (**B**) values in skeletal muscle in ovalbumin (*OVA*), control (CON), methylene blue (MB), epinephrine (EPI), and epinephrine associated with methylene blue (MB–EPI) groups ($n = 6$ rats/group). Because of the microdialysis flow rate, samples were collected every 10 mins, since 10 mins before shock induction (T0). OVA rats died within 15 mins, so the data were not measured. Values are presented as mean ± SEM. ↑ corresponds to injection of treatment bolus. $^{\#}p \leq 0.05$ between-group differences MB–EPI or EPI or MB groups vs. CON group. Triangle = OVA group; white square = CON group; black square = MB group; white circle = EPI group; black circle = MB-EPI group; $mPtiO_2$ = muscular oxygen partial pressure.

L/P ratios was observed in all treatment groups; values were significantly different in the group MB alone and EPI alone as compared with the CON group (Fig. 3*B*). However, maximal values obtained at the end of the experiment tended to be lower in the MB–EPI-treated animals, although this difference failed to reach statistical significance among treatment groups.

Time Course of Systemic Biochemistry Variables

The time course of the main systemic biochemistry variables is summarized in **Table 3**. No differences were observed among groups before OVA injection (T0). Data from the OVA group are not shown because of animals' death after 15 mins, and so biochemistry results were not measured. Sixty mins after onset of shock, no significant differences were observed among treated groups, except for $PaCO_2$, nitrate, and nitrite. In each group, AS induced a severe lactic acidosis and hemoconcentration (hemoglobin: from 12.1 ± 0.3 to 14.6 ± 0.4 g/dL).

Expression of Activated Caspase 3 and HIF-1α in the Cerebral Cortex

Sixty mins after the onset of shock, there was a significant increase in the expression of activated caspase 3 in the cerebral cortex in the MB and EPI groups compared with the MB–EPI group. It was moderately underexpressed in the MB–EPI group compared with the CON group but not significantly. With regard to HIF-1α, changes were not significantly different among groups. It was underexpressed in the MB and EPI groups and similar to the CON group in the MB–EPI group (**Fig. 4**).

DISCUSSION

According to our knowledge, this is the first report describing the highly deleterious consequences of AS on the brain and their attenuation by three different treatment regimens that are routinely (EPI) or potentially (MB) used in humans who have AS.

Under physiological conditions, the cerebral circulation is intimately involved in adequate blood distribution (19–22). Maintenance of CBF during hypotension is essential for homeostasis and prevention of irreversible brain damage. It is assumed, but not demonstrated, that even in severe AS states, cerebral circulation is maintained (11). Meanwhile, a severe decrease in regional flow is expected to occur in skeletal muscle. Our results demonstrate that in untreated AS, the dramatic decrease in CO and CBF precluded any redistribution of blood flow to the brain and resulted in severe brain hypoxia and metabolic disturbances (Figs. 1*B* and 2*A*).

Concerning the pharmacological interventions that could attenuate the decrease in CBF and its deleterious cerebral consequences, the main findings of this study on the treatment of AS were as follows:

1. In this model of lethal AS, administration of MB alone extended survival time but did not prevent neuronal injury, as demonstrated by a major glutamate release; this is probably the result of poor improvement of hemodynamic parameters and tissue perfusion. Therefore, MB alone cannot be recommended as monotherapy.
2. EPI, the recommended treatment of AS, partially restored systemic hemodynamic variables and cerebral perfusion, thus preventing glutamate-induced excitotoxicity.
3. The MB–EPI association, just like EPI alone, avoided neuronal excitotoxicity and had an additive effect on hemodynamic variables and for the prevention of brain ischemia and neuronal apoptosis compared with individually administered drugs.
4. Neither treatment significantly restored CO nor prevented muscular compartment ischemia and microvascular leakage.

A key reason for studying the effect of MB in AS is the suggestion that MB might be a potential therapeutic drug in catecholamine-refractory vasoplegia associated with sepsis and the systemic inflammatory reaction syndrome in a variety of clinical contexts including cardiac surgery (23–25).

In addition, several clinical case reports have been published supporting the clinical interest of MB in AS. In most cases, MB was administered either as a bolus or as a continuous infusion in combination with EPI (14). In our study, we used a single 3-mg/kg bolus of MB, similar to the therapeutic doses, which have been reported as safe and efficient in other types of refractory hypotension (26–28). Although doses as high as 5

TABLE 2. Time Course of Interstitial Lactate and Pyruvate Concentrations in the Skeletal Muscle ($n = 6$ rats/group)

Group		Control Group	Methylene Blue Group	Epinephrine Group	Epinephrine Associated With Methylene Blue Groups
Lactate (mM)	T10 to T0	0.37 ± 0.04	0.38 ± 0.12	0.69 ± 0.14	1.13 ± 0.17[a,b]
	T0 to T10	0.42 ± 0.03	0.47 ± 0.13	0.63 ± 0.12	0.98 ± 0.16[a,c]
	T10 to T20	0.48 ± 0.05	0.79 ± 0.13	0.92 ± 0.13	1.63 ± 0.22[a,b,c]
	T20 to T30	0.43 ± 0.05	1.20 ± 0.18[c]	1.64 ± 0.22[c]	2.13 ± 0.22[a,c]
	T30 to T40	0.47 ± 0.04	1.72 ± 0.29[c]	2.59 ± 0.41[c]	2.29 ± 0.25[c]
	T40 to T50	0.46 ± 0.04	1.91 ± 0.31[c]	2.35 ± 0.32[c]	2.26 ± 0.25[c]
	T50 to T60	0.48 ± 0.04	2.46 ± 0.53[c]	2.70 ± 0.39[c]	2.35 ± 0.56[c]
Pyruvate (mM)	T10 to T0	0.028 ± 0.003	0.047 ± 0.011	0.056 ± 0.014	0.078 ± 0.012
	T0 to T10	0.036 ± 0.004	0.052 ± 0.013	0.055 ± 0.011	0.070 ± 0.013[c]
	T10 to T20	0.036 ± 0.010	0.049 ± 0.011	0.056 ± 0.011	0.073 ± 0.010[c]
	T20 to T30	0.037 ± 0.006	0.043 ± 0.012	0.056 ± 0.007	0.075 ± 0.012[c]
	T30 to T40	0.043 ± 0.006	0.057 ± 0.021	0.053 ± 0.007	0.068 ± 0.010
	T40 to T50	0.045 ± 0.005	0.043 ± 0.012	0.051 ± 0.008	0.057 ± 0.009
	T50 to T60	0.044 ± 0.004	0.033 ± 0.005	0.041 ± 0.010	0.055 ± 0.007[a]

Time 0 (T0) corresponds to injection of ovalbumin.
[a]$p \leq 0.05$ vs. methylene blue group.
[b]$p \leq 0.05$ vs. epinephrine group. [c]$p \leq 0.05$ vs. control group.

to 7.5 mg/kg are usually well tolerated (14), the use of doses of ≤4 mg/kg has been recommended (29), the 50% lethal dose in sheep being estimated at 40 mg/kg (30).

In the present investigations, a single bolus of MB significantly enhanced survival time but stabilized hemodynamic variables at low values as compared with baseline. This confirms results published by other groups (17). In our experiments, treatment with MB alone did not prevent cerebral ischemia attested by decreased $cPtiO_2$ values and neuronal injury, demonstrated by the major glutamate release observed in microdialysate samples. Although clinically insufficient, these results clearly support the interest in NO pathway modulation and the use of MB for the treatment of severe AS, as reported by other groups (17, 31). Indeed, several studies conducted in different species have demonstrated that NO is an important mediator in anaphylaxis. However, the beneficial vs. detrimental effects on hemodynamics, organ dysfunction, and survival associated with modulation of the NO pathway remain unclear.

Studies conducted in mice models reported beneficial effects of N^G-nitro-L-arginine-methyl ester administration on both hypotension and survival (14, 16, 32) and demonstrated the role played by endothelial NO synthase in murine anaphylaxis (13). On the contrary, MB only partially prevented PAF-induced shock (13) or failed to prevent hypotension in OVA-induced shock (18), suggesting that effects of NO, independent from soluble guanylyl cyclase activation, might play a role.

However, studies conducted in rabbits reported decreased survival resulting from worsened bronchospasm and more severe cardiac depression (33). Similar results were observed in IgE-mediated anaphylaxis in dogs, in which N-nitro-L-arginine methyl ester, a nonselective inhibitor of NO synthase isoforms, corrected the vasodilatation and hemoconcentration but did not improve cardiac function (34). This was also reported by our group in this model of anaphylaxis in Brown-Norway rats, showing that constitutive NO synthase inhibition combined with histamine and serotonin receptor blockade improved the initial OVA-induced arterial hypotension but not survival (35). In contrast, 7-nitroindazole, a putative selective NO synthase inhibitor, but not L-NAME or aminoguanidine, has been shown to attenuate arterial hypotension during AS in rats (36). Finally, as observed in the present study, improved MAP and prolonged survival have been reported with the use of MB during experimental anaphylaxis in rabbits (17).

Part of the differences observed might be species-specific. It is probable, but this requires demonstration, that the degree of cardiac dysfunction in different models of AS can also play a role. Extrapolating from experimental models of sepsis and from human studies, it is conceivable that in the presence of severe myocardial dysfunction, NO synthase inhibitors may worsen survival by further alteration of right ventricular function through increased right ventricular afterload (37). From a clinical point of view, all these results are not an incentive to the use of MB alone to treat AS mainly because of reduced

TABLE 3. Biochemistry Variables in Control, Methylene Blue, Epinephrine, and Epinephrine Associated With Methylene Blue Groups (n = 12 rats/group)

Group	Time (mins)	Control Group	Methylene Blue Group	Epinephrine Group	Epinephrine Associated With Methylene Blue Groups
pH	T0	7.44 ± 0.02	7.41 ± 0.02	7.42 ± 0.01	7.42 ± 0.01
	T60	7.44 ± 0.02	7.16 ± 0.07[a,b]	7.17 ± 0.04[a,b]	7.09 ± 0.04[a,b]
P_{CO_2} (mm Hg)	T0	27.2 ± 1.7	28.5 ± 1.7	29.9 ± 1.1	30.2 ± 1.2
	T60	29.3 ± 1.4[c]	17.0 ± 2.0[a,b]	27.0 ± 4.0[c]	31.8 ± 4.3[c]
P_{O_2} (mm Hg)	T0	254.5 ± 18.3	283.3 ± 19.4	294.0 ± 12.4	269.3 ± 15.3
	T60	272.2 ± 12.0	289.5 ± 39.2	287.4 ± 28.2	228.8 ± 31.1
H_{CO_3} (mmol/L)	T0	17.8 ± 0.7	18.3 ± 0.7	19.3 ± 0.5	18.9 ± 0.4
	T60	19.4 ± 0.6	7.0 ± 1.7[a,b]	9.1 ± 0.7[a,b]	9.2 ± 1.0[a,b]
Lactate (mmol/L)	T0	2.7 ± 0.2	2.5 ± 0.2	2.6 ± 0.3	2.7 ± 0.2
	T60	3.2 ± 0.3	8.3 ± 0.8[a,b]	7.0 ± 0.5[a,b]	7.6 ± 0.7[a,b]
Hemoglobin (g/dL)	T0	12.3 ± 0.4	12.1 ± 0.3	12.9 ± 0.2	12.5 ± 0.2
	T60	12.0 ± 0.3	14.6 ± 0.4[b]	14.9 ± 0.5[b]	15.2 ± 0.5[b]
Glucose (mmol/L)	T0	13.6 ± 0.4	12.1 ± 1.2	12.5 ± 0.7	14.2 ± 1.1
	T60	10.1 ± 0.5	11.8 ± 1.5	13.8 ± 1.6	15.7 ± 2.0[a]
Sodium (mmol/L)	T0	137.2 ± 1.2	137.8 ± 0.9	136.4 ± 0.5	136.6 ± 0.6
	T60	137.0 ± 0.6	138.9 ± 1.9	137.9 ± 1.0	131.4 ± 1.4
Nitrite (μmol/L)	T60	2.4 ± 0.8	4.1 ± 1.4	4.1 ± 0.9	8.7 ± 1.5[a,c,d]
Nitrate (μmol/L)	T60	28.4 ± 1.7	22.4 ± 2.5[a]	28.0 ± 1.6[c]	31.7 ± 1.7[c]

Time 0 corresponds to the injection of ovalbumin. All biochemistry variables were measured in plasma. Values are presented as mean ± SEM. To avoid massive blood spoiling before injection of ovalbumin, we measured nitrite and nitrate only at the end of the experiment.

[a]$p \leq 0.05$ vs. control group.

[b]$p \leq 0.05$ vs. time 0.

[c]$p \leq 0.05$ vs. methylene blue group.

[d]$p \leq 0.05$ vs. epinephrine group.

effectiveness. Furthermore, direct inhibitors of NO synthase isoforms are not available in clinical practice.

As could be expected, EPI, the recommended treatment of AS, restored partially systemic hemodynamic variables and cerebral perfusion thus preventing cerebral ischemia and glutamate-induced excitotoxicity as demonstrated by our brain microdialysis results. However, EPI failed to normalize cerebral oxygen availability and could not prevent a significant increase in caspase 3 and a trend in increased HIF-1α expression. This increase in HIF-1α expression failed to reach statistically significant values, probably because of the early tissue sampling time in this study (38).

Interestingly, in this model of lethal anaphylaxis, the combination of EPI and MB was significantly more effective than EPI alone. Although this association failed to normalize CO values, it restored MAP, CBF, and brain oxygen availability. It also prevented any increase in microdialysis L/P ratio and glutamate concentrations as well as caspase 3 and HIF-1α expression arguing against the presence of severe ischemic brain injury. Several hypotheses might explain this apparent synergistic effect. Apart from the direct hemodynamic effects of both drugs and the interaction between NO and catecholamines classically described (39–41), other factors directly related to concomitant MB and EPI administration might also be involved.

Indeed, the discordance between the improved survival associated with MB alone and the lack of correction of tissue perfusion and abnormalities can be related to the NO pathway-independent effects of MB. After systemic administration, MB can be rapidly and extensively accumulated in the nervous system (42). It has been used as a neuroprotective agent in drug-induced encephalopathy, dementia, and manic–depressive psychosis (38, 43, 44). Furthermore, MB exhibits promising cardio- and neuroprotective properties in experimental cardiac arrest and is effective in both attenuating ischemia–reperfusion syndrome and increasing short-term survival after resuscitation (45, 46). MB exerts neuroprotection by regulation of the expression of soluble guanylyl cyclase and diverse biological processes ranging

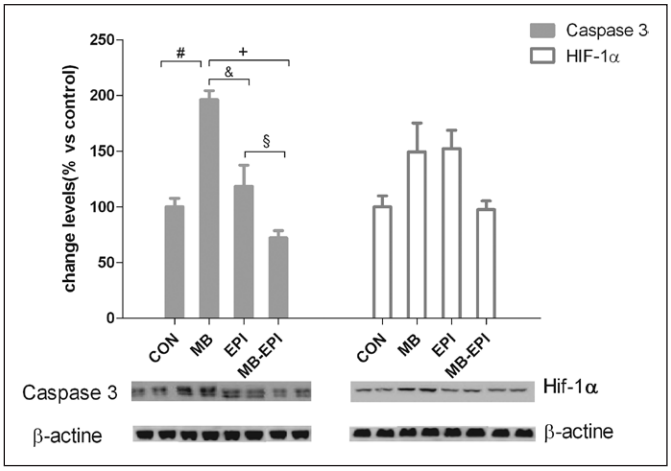

Figure 4. Expression of activated caspase 3 (*gray column*) and hypoxia-inducible factor (HIF)-1α (*white column*) in the cerebral cortex in control (CON), methylene blue (MB), epinephrine (EPI), and epinephrine associated with methylene blue (MB–EPI) groups ($n = 6$ rats/group). Because all rats in the ovalbumin group died within 15 mins, tissues were not used. $^{+}p \leq 0.05$ between-group differences MB–EPI group vs. MB group, $^{\&}p \leq 0.05$ between-group differences EPI group vs. MB group, $^{\S}p \leq 0.05$ between-group differences MB–EPI group vs. EPI group, $^{\#}p \leq 0.05$ between-group differences MB group vs. CON group.

from inhibition of apoptosis and reversal of the shutdown of translation to restoration of functional cellular trafficking and activation of brain repair/regeneration genes as well as induction of critical neuroprotective proteins (47).

Another important finding of our study concerns the discordance between correction of arterial hypotension and persistence of a very low CO after treatment of AS. The consequences of this discordance are illustrated by the persistence of muscular and brain hypoxia with its deleterious metabolic consequences (increased lactate/pyruvate ratio and glutamate release). This observation suggests that on partial or complete correction of arterial hypotension, one should anticipate persistence of low CO. Furthermore, in cases of AS refractory to conventional treatment (lack of correction of MAP), a very low CO may continue and place patients at risk for severe brain dysfunction. This could be attributable to severe hypovolemia (demonstrated in our experiments by hemoconcentrations), a plausible cause in patients without prior cardiac disease, or severe cardiac dysfunction. In such cases, therapeutic measures other than EPI should be considered.

Limitations of the Study

In the present study, we did not observe any alterations in the plasma nitrite level after the different treatment regimens. These results are consistent with previous reports. NO production can be detected in tissue using an NO-sensitive electrode in anaphylactic rabbits (33), but not in plasma (17). A lack of increase in plasma nitrate concentrations has also been reported in 48–80 induced AS in pigs (23). Similar results have been obtained in men, in whom increased NO production has been detected in exhaled breath (48), whereas no significant increase in NO levels was detected in plasma (49). Taken together, these results suggest that NO production can be detected in tissue but not in plasma.

Another potential limitation is the absence of a high level of volume expansion, which was limited to the injection of a bolus of 1 mL of saline followed by a continuous infusion at a dose of 10 mL/kg/hr. Further studies are required to investigate. The additional effect of various volume expansion protocols in our model of lethal anaphylaxis remains to be investigated.

Further studies are required to identify the mechanisms underlying the potential beneficial effects of the MB–EPI association. Although addition of MB in cases of AS refractory to conventional treatment cannot be recommended, such an intervention may be considered in difficult cases.

CONCLUSIONS

Our results suggest that association of MB to EPI but not MB alone may have beneficial effects in severe AS through mechanisms that remain to be elucidated. Furthermore, on treatment of AS, the dissociation between corrected MAP and persistently low CO continues to place the subject at risk for systemic and neurologic hypoperfusion because brain ischemia, hypoxia, and metabolic dysfunction are major characteristics of AS.

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