Effects of bupivacaine on human erythrocytes submitted to stress and evidence for an interaction between bupivacaine and flumazenil

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Aims To examine the effects of bupivacaine on erythrocytes submitted to an oxidative stress (AAPH) and to provide evidence for an *in vitro* interaction between bupivacaine and flumazenil.

Methods Human erythrocytes were studied with or without AAPH in the presence of different concentrations of bupivacaine (0.15, 0.3, 0.9 and 1.8 mmol l⁻¹), or flumazenil (0.16 mmol l⁻¹) and with the association of flumazenil and two doses of bupivacaine (0.15 and 0.3 mmol l⁻¹). Potassium efflux was measured by flame photometry at *t*₀, and every 30 min for 2 h.

Results In the absence of AAPH, extracellular potassium remained unchanged. Oxidative stress induced a significant increase in extracellular potassium, which was not modified by incubation with flumazenil. Bupivacaine significantly lowered the increase in extracellular potassium in a dose-related fashion. The association with flumazenil blunted the effects of bupivacaine.

Discussion In this model, bupivacaine proved effective in protecting erythrocytes against oxidative stress. Flumazenil interacted with bupivacaine and blunted its protective effects.

Keywords: bupivacaine, drug interaction, erythrocytes, flumazenil, oxidative stress

Introduction

Recently we reported that flumazenil was able to correct the bupivacaine-induced intracardiac conduction abnormality in a patient who received accidental intravenous injection of a toxic dose of bupivacaine [1]. Moreover, experimental *in vivo* study suggest that flumazenil strongly modifies the local anaesthetic-induced toxicity in mice [2]. The mechanisms of such interaction remain unclear. The aim of this study was to examine the effect of bupivacaine on erythrocytes submitted to oxidative stress and to study, under such conditions, the interaction between bupivacaine and flumazenil.

Methods

Erythrocytes, obtained from blood samples from five healthy volunteers were washed five times in isotonic

saline solution, centrifuged and suspended in phosphate buffer (pH 7.4).

Oxidative stress was induced by addition of 2, 2' azobis (2-amidinopropane) hydrochloride (AAPH) (Interchim, Montluçon, France) at 20 mM.

Experiments were performed according to the following design. The control groups consisted of erythrocytes in the absence of AAPH and in the presence of AAPH. The test groups were erythrocytes containing bupivacaine (0.15, 0.3, 0.9 and 1.8 mM (50, 100, 300 and 600 mg l⁻¹), respectively) in the absence of AAPH, or in the presence of AAPH, erythrocytes containing flumazenil (0.16 mM (50 mg l⁻¹)) in the absence of AAPH or in the presence of AAPH and erythrocytes containing bupivacaine (0.15 and 0.3 mM) and flumazenil (0.16 mM) in the absence of AAPH or in the presence of AAPH. Experiments were performed separately on erythrocytes from the five subjects.

Racemic bupivacaine without preservative (Marcaine 0.5%, ASTRA Laboratory) and racemic flumazenil (Anexate, ROCHE Laboratory) were used. The concentrations of bupivacaine were chosen after a preliminary study in our laboratory to determine the minimum concentration showing an effect on potassium efflux.

A preliminary report was presented at the 1996 Annual Meeting of the European Society of Anaesthesiology.

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Received 9 December 1998, accepted 8 August 1999.

These concentrations were similar to plasma concentrations reported in clinical practice [3] and to the concentrations studied in an *in vitro* model [4]. The concentration of flumazenil was chosen according to published *in vitro* data [5] but was greater than plasma concentrations in therapy [6].

Samples were incubated at 37° C and the pH of each sample was controlled at the beginning and at the end of the experiment. The concentration of extra cellular potassium was measured by flame photometry (Flame Photometer 410, CIBA Corning) at time=0 and every 30 min for 2 h.

Data analysis

Areas under the potassium concentration *vs* time curves (AUC) were calculated and expressed in arbitrary units. Data are expressed as mean $\pm$ s.d. and [95% confidence interval]. An analysis of variance was used to compare the different groups and followed by a Tukey test. $P < 0.05$ was considered as significant.

Results

The pH of the different incubates varied from 7.2 to 7.4. When erythrocytes were incubated in the absence of AAPH, no potassium efflux was noted (data not shown). Oxidative stress in the presence of AAPH induced a marked increase in potassium AUC (1404 ± 207 arbitrary units), which was unaffected by flumazenil (1427 ± 242 arbitrary units). In the presence of bupivacaine, potassium AUCs were significantly lowered (1117 ± 250 , 1003 ± 212 , 777 ± 97 and 730 ± 36 arbitrary units at 0.15, 0.3, 0.9 and 1.8 mM, respectively) (Figure 1). Addition of flumazenil counteracted the effects of bupivacaine 0.15 and 0.3 mM (1414 ± 69 and 1392 ± 42 arbitrary units, respectively) (Figure 2).

Discussion

Incubation of erythrocytes with AAPH induces haemolysis and potassium efflux, both of which constitute good indicators of oxidative stress induced alterations of erythrocytic membrane [7, 8]. Pretreatment of erythrocytes subject to oxidative stress with bupivacaine caused a marked decrease in extracellular potassium concentration, that tended to be concentration-dependent. Thus, we conclude that bupivacaine is protective against oxidative stress in this model. Local anaesthetic agents have membrane stabilizing properties which may induce changes in membrane polarization [9]. This mechanism may account for the protective effect of bupivacaine against oxidative stress. Flumazenil was found to counteract completely this action of bupivacaine. Such an

Figure 1 Areas under the potassium concentration *vs* time curves expressed in arbitrary units when erythrocytes are incubated with AAPH (A) (20 mM) and with 0.15, 0.3, 0.9 or 1.8 mM of bupivacaine (AB50, AB100, AB300 and AB600, respectively). The results are expressed as mean $\pm$ s.d. values from the blood of five subjects. * $P < 0.05$ *vs* A.

Figure 2 Areas under the potassium concentration *vs* time curves expressed in arbitrary units when erythrocytes were incubated with AAPH (A) (20 mM) and bupivacaine at the concentration of 0.15 mM (AB50) or 0.3 mM (AB100) or with flumazenil (0.16 mM) (AF50) and the two previous concentrations of bupivacaine (AF50B50 and AF50B100, respectively). The results are expressed as mean $\pm$ s.d. values from the blood of five subjects. * $P < 0.05$ *vs* A.

interaction between flumazenil and bupivacaine may involve a modification of the free radical scavenging properties of bupivacaine although one report indicates that bupivacaine is not a free radical scavenger [10]. Another possibility is because bupivacaine acts only in its ionized form and has a pKa of 8.1, a modification of the pH induced by the addition of flumazenil could account for this interaction. However, the pH of the solutions was stable and we consider that it did not induce significant changes in the percentage of the ionized form of bupivacaine. A further possible mechanism for such an interaction consists in the modification of the binding of bupivacaine to its receptor. *In vivo* experiments suggest that bupivacaine kinetics were not modified by flumazenil [11], but in our experimental conditions, a decrease in bupivacaine affinity for its receptor induced by the flumazenil cannot be ruled out.

This *in vitro* study has demonstrated an interaction between bupivacaine and flumazenil, affecting the response of human erythrocytes to oxidative stress. These data are consistent with previous reported *in vivo* findings in which the toxicity of bupivacaine was modified by flumazenil [2]. Moreover, this study confirms the evidence for an interaction between flumazenil and bupivacaine that could be of importance in clinical practice [1]. There is a need for further experiments in order to explain the mechanisms of such an interaction.

This study was performed at the Laboratoire de Physiopathologie et Pharmacologie Cardiovasculaires expérimentales, Faculté de Médecine de l'Université de Bourgogne, 7 Boulevard Jeanne d'Arc, 21033 Dijon Cedex, France.

The authors gratefully acknowledge the Conseil Regional de Bourgogne for its continuing support.

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