Molecular Genetics and Metabolism **69,** 40–45 (2000)
doi:10.1006/mgme.1999.2954, available online at http://www.idealibrary.com on IDEAL®

Local Anesthetics Induce a Decrease in the Levels of Glucose 1,6-Bisphosphate, Fructose 1,6-Bisphosphate, and ATP, and in the Viability of Melanoma Cells

Malka Karniel and Rivka Beitner[1]

Health Sciences Research Center, Faculty of Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel

Received November 23, 1999

Glycolysis is known to be the primary energy source in cancer cells. We investigated here the effect of local anesthetics, lidocaine and bupivacaine, on the levels of glucose 1,6-bisphosphate and fructose 1,6-bisphosphate, the two stimulatory signal molecules of glycolysis, and on ATP levels and cell viability in B16 melanoma cells. We found that both drugs induced a significant, dose-dependent reduction in the levels of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate, ATP, and cell viability. Bupivacaine was more potent than lidocaine. The decrease in glucose 1,6-bisphosphate and fructose 1,6-bisphosphate, induced by the local anesthetics, preceded the reduction in the viability of melanoma cells, indicating that these are early changes and not a result of cell death. Cell viability was reduced in a close correlation with the fall in ATP. These findings suggest that the fall in the levels of the two signal allosteric regulators of glycolysis, induced by the local anesthetics, is one of the mechanisms that causes a reduction in glycolysis and ATP levels, which eventually leads to melanoma cell death. These experiments suggest that local anesthetics, and especially bupivacaine, are most promising agents in the treatment of melanoma.

Key Words: **local anesthetics; melanoma; glucose 1,6-bisphosphate; fructose 1,6-bisphosphate; ATP; bupivacaine; lidocaine; glycolysis.**

Cancer cells are known to exhibit a high rate of glycolysis, even under aerobic conditions, which is their primary energy source (1–4). One of the important mechanisms that control glycolysis is through allosteric regulators, such as glucose 1,6-bisphosphate and fructose 1,6-bisphosphate (for reviews, see 5–10).

We have previously found that calmodulin antagonists, and especially clotrimazole and bifonazole (the antifungal drugs with calmodulin antagonistic activity), are most effective in treatment of melanoma. They act by reducing glycolysis, which eventually leads to melanoma cell death (11–13; for review, see 10). An important mechanism of their action is the reduction in the levels of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate, and ATP (11). We have recently found that this mechanism is also involved in the action of taxol on melanoma cells (14). These results suggest that this may be a general mechanism involved in the action of anticancer drugs.

In our early studies in normal muscle (15,16), we found that local anesthetics induced a marked decrease in the level of glucose 1,6-bisphosphate. Concomitant to the decrease in glucose 1,6-bisphosphate, the powerful allosteric activator of phosphofructokinase (5,17) (the rate-limiting enzyme of glycolysis), the activity of this enzyme, and thereby glycolysis and ATP level were significantly reduced. These changes induced by the local anesthetics closely resembled the changes we found in muscular dystrophy (18,19), or tissue damage (for review, see 10). These experiments prompted us to investigate the effect of local anesthetics on melanoma cells.

We report here that the local anesthetics, lido-

[1] To whom correspondence should be addressed at Faculty of Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel. Fax: 972-3-5351824. E-mail: beitnr@mail.biu.ac.il.

1096-7192/00 $35.00

caine and bupivacaine, decreased the levels of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate, and ATP and the viability of B16 melanoma cells.

MATERIALS AND METHODS

Materials. Lidocaine and bupivacaine were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals and enzymes were either from Sigma Chemical Co. or from Boehringer Mannheim (Mannheim, Germany). Tissue culture reagents were purchased from Biological Industries (Beit Haemek, Israel).

Cell culture. B16 F10 mouse melanoma cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics, at 37°C in humidified atmosphere at 5% CO_2 and 95% air. Cells were passaged two to three times weekly.

Treatment of culture. Melanoma cells (9×10^{-5} cells ml^{-1}) were seeded in tissue culture plates (10 cm). Twenty-four hours before the experiment, the fetal calf serum was removed and the cells were grown in serum-free medium. The cells were incubated at 37°C in RPMI 1640 medium in the absence and presence of local anesthetics at different concentrations. Lidocaine was dissolved in RPMI 1640 medium. Bupivacaine was dissolved in warm water. The appropriate solvents were added to the controls.

Extraction of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate, and ATP. The cells were washed twice with ice-cold RPMI and placed on ice and 1.5 ml HCl 0.05 N was added to the dish. Ten minutes later, the cells were scraped with a rubber policeman and collected in a centrifuge tube at 0°C. Samples (10 μl) were removed for protein determination. Cells were mixed with 120 μl of 70% cold perchloric acid containing 130 mM EDTA. After 5 min, the extract was neutralized with KOH. The precipitated potassium perchlorate was removed by centrifugation at 4°C for 15 min at 5000*g*, and the clear supernatant was used for determination of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate, and ATP.

Measurements of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate, and ATP levels. Glucose 1,6-bisphosphate was measured by the fluorometric method of Passonneau *et al.* (20); glucose 1,6-bisphosphate, being a cofactor of phosphoglucomutase reaction, was coupled with glucose-6-phosphate dehydrogenase and $NADP^+$. The buffer consisted of 50 mM imidazole-HCl, pH 7, containing 1 mM magnesium acetate, 0.1 mM EDTA, and 0.01% bovine serum albumin. First phosphoglucomutase (4 U/ml) was activated in the buffer for 1 h. The reaction mixture contained 15 μM glucose 1-phosphate, 2 mU/ml of activated phosphoglucomutase, 0.05 mM $NADP^+$, and 1 U/ml glucose-6-phosphate dehydrogenase in 1 ml buffer. The reaction was started by the addition of 2–50 μl from the extract. Calculation was made from a standard of the glucose 1,6-bisphosphate between 10^{-8} and 5×10^{-9} M.

Fructose 1,6-bisphosphate and ATP were measured by the method of Lowry *et al.* (21).

Cell viability determination. After incubation in the absence and presence of local anesthetics, the cells were harvested with trypsin (0.25%)-EDTA (0.05%) and centrifuged for 10 min at 270*g*. The precipitated cells were suspended in PBS and counted in hemocytometer (Neubauer). Cell viability was determined by trypan blue dye exclusion.

Protein measurement. Protein was measured by the method of Bradford (22) with crystalline bovine serum albumin as a standard.

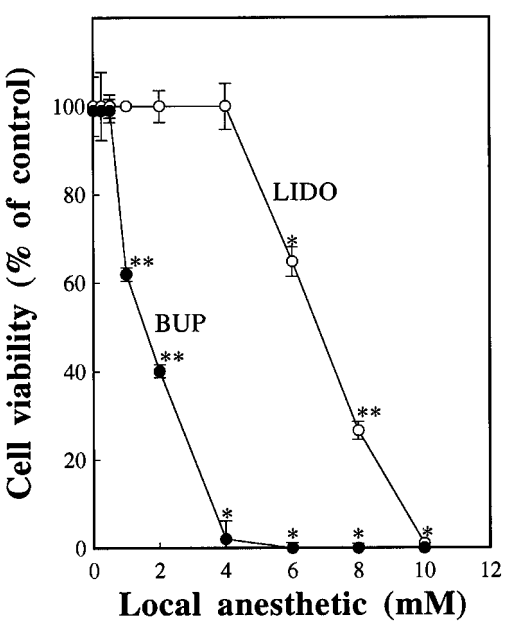

FIG. 1. Dose-response curves of the effects of the local anesthetics, lidocaine (LIDO) and bupivacaine (BUP), on cell viability of B16 melanoma cells. Cells were incubated for 20 min in the absence and presence of different concentrations of local anesthetics. 100% cell viability refers to 5×10^6 cells ml^{-1}. Each point is the mean ± SEM of 2–3 separate experiments which were performed in triplicate. $^{**}P < 0.005$, $^{*}P < 0.05$.

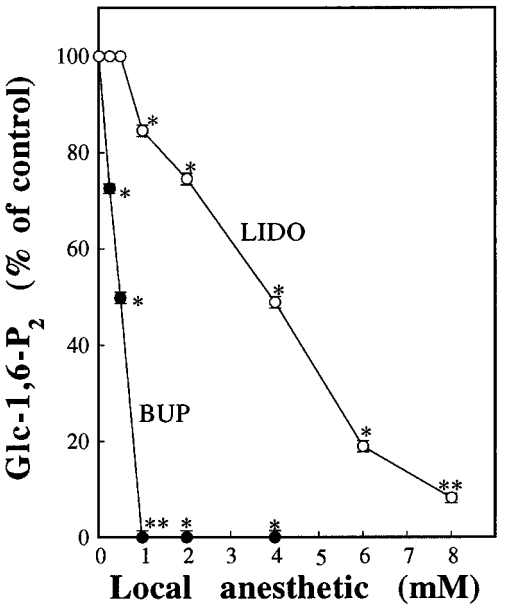

FIG. 2. Dose-response curves of the effects of the local anesthetics, lidocaine (LIDO) and bupivacaine (BUP), on glucose 1,6-bisphosphate (Glc-1,6-P_2) levels in B16 melanoma cells. Cells were incubated for 20 min with and without the local anesthetics. 100% glucose 1,6-bisphosphate refers to 0.366 ± 0.015 (nmol/mg protein). Each point is the mean ± SEM of 2–3 separate experiments which were performed in triplicate. $^{**}P < 0.005$, $^{*}P < 0.05$.

RESULTS

The results presented in Fig. 1 show the effect of the local anesthetics, lidocaine and bupivacaine, on the cell viability of B16 melanoma cells. It can be seen that both compounds induced a rapid, concentration-dependent reduction in cell viability. Bupivacaine was more potent than lidocaine, decreasing the cell viability almost totally at a concentration of 4 mM, whereas 10 mM lidocaine was required to induce a similar effect.

Figure 2 shows that both local anesthetics exerted a dose-dependent decrease in the levels of glucose 1,6-bisphosphate. Here again the action of bupivacaine was more pronounced. Similarly, both local anesthetics also reduced the levels of fructose 1,6-bisphosphate (Fig. 3) and ATP (Fig. 4) in melanoma cells, in a concentration-dependent manner.

The results presented in Figs. 5 and 6 show that the decrease in glucose 1,6-bisphosphate and fructose 1,6-bisphosphate occurred at lower concentrations of the drugs than those required to decrease cell viability. Figures 5 and 6 also reveal that there was a close relationship between ATP levels and cell viability.

DISCUSSION

The present results reveal that the local anesthetics, lidocaine and bupivacaine, induced a dose-dependent decrease in the levels of glucose 1,6-bisphosphate in B16 melanoma cells (Fig. 2). Glucose 1,6-bisphosphate is the chief allosteric regulator of cytosolic glycolysis in extrahepatic tissues (for reviews, see 5–9). Glucose 1,6-bisphosphate is a potent allosteric activator of phosphofructokinase, the rate-limiting enzyme of glycolysis (5,17). The decrease in glucose 1,6-bisphosphate induced by the local anesthetics causes a reduction in the activity of phosphofructokinase, as reflected here by the drastic fall in fructose 1,6-bisphosphate (Fig. 3), which is the product of phosphofructokinase reaction and also an allosteric activator of this enzyme. Fructose 1,6-bisphosphate levels were found to be elevated in cancer cells (1). Both glucose 1,6-bisphosphate and fructose 1,6-bisphosphate are allosteric activators of pyruvate kinase, another key enzyme in glycolysis

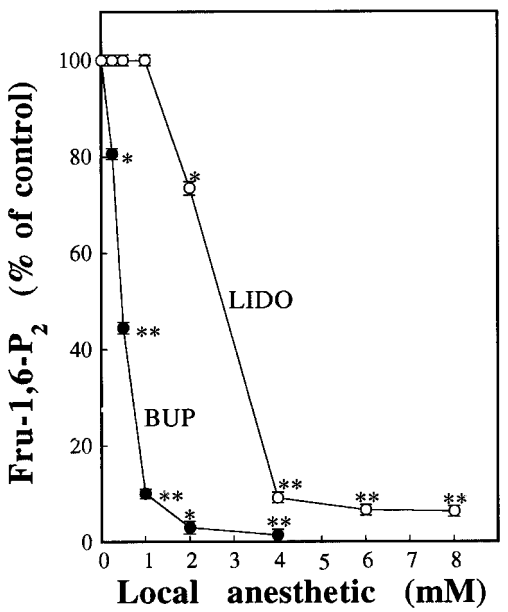

FIG. 3. Dose-response curves of the effects of the local anesthetics, lidocaine (LIDO) and bupivacaine (BUP), on fructose 1,6-bisphosphate (Fru-1,6-P_2) levels in B16 melanoma cells. Cells were incubated for 20 min with and without the local anesthetics. 100% fructose 1,6-bisphosphate refers to 21.33 ± 1.20 (nmol/mg protein). Each point is the mean ± SEM of 2–3 separate experiments which were performed in triplicate. $^{**}P < 0.005$, $^{*}P < 0.05$.

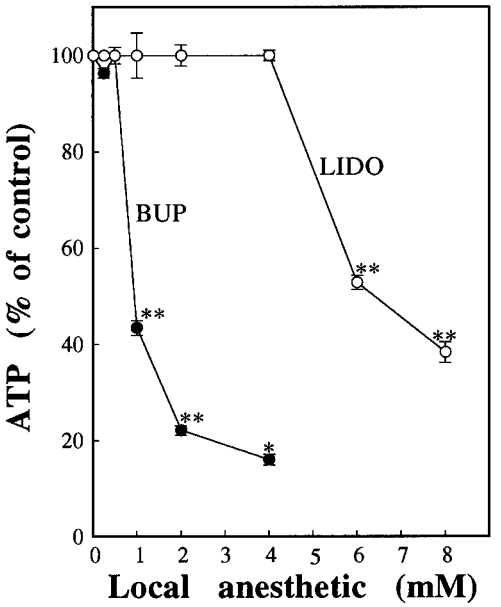

FIG. 4. Dose-response curves of the effects of the local anesthetics, lidocaine (LIDO) and bupivacaine (BUP), on ATP levels in B16 melanoma cells. Cells were incubated for 20 min with and without the local anesthetics. 100% ATP refers to 44.75 ± 1.52 (nmol/mg protein). Each point is the mean ± SEM of 2–3 separate experiments which were performed in triplicate. $^{**}P < 0.005$, $^{*}P < 0.05$.

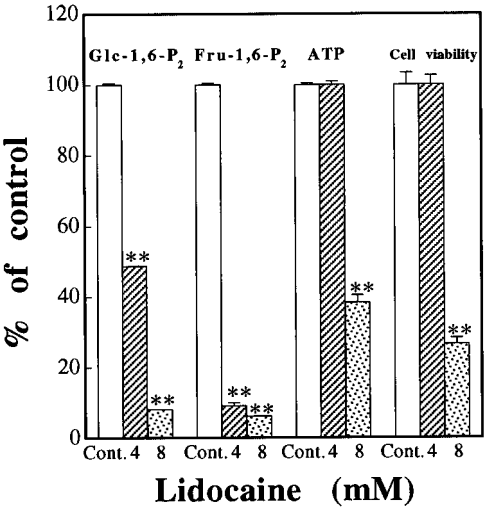

FIG. 5. Effect of lidocaine on cell viability and its relation to the levels of ATP, glucose 1,6-bisphosphate (Glc-1,6-P_2), and fructose 1,6-bisphosphate (Fru-1,6-P_2). Cells were incubated with and without 4 and 8 mM lidocaine for 20 min. 100% cell viability refers to 5×10^6 cells ml^{-1}; 100% ATP, glucose 1,6-bisphosphate, and fructose 1,6-bisphosphate levels refer to 44.75 ± 1.52, 0.366 ± 0.015, and 21.33 ± 1.20 (nmol/mg protein), respectively. Values are the means ± SEM of 2–3 separate experiments which were performed in triplicate $^{**}P < 0.005$.

(for reviews, see 5,7). The decrease in both these allosteric stimulatory signal molecules of glycolysis, induced by the local anesthetics, would lead to a reduction in ATP production through cytosolic glycolysis. Other experiments in our laboratory (unpublished results) have revealed that the local anesthetics also reduced cytoskeletal glycolysis by causing a detachment of glycolytic enzymes from cytoskeleton of B16 melanoma cells. In Ehrlich ascites tumor cells, bupivacaine was also reported to decrease hexokinase activity, especially the mitochondria-bound enzyme (23). Hexokinase is the only glycolytic enzyme which does not bind to cytoskeleton, but instead binds reversibly to mitochondria, where it is linked to oxidative phosphorylation (24–27; for review, see 28,29). Local anesthetics were reported to reduce mitochondrial oxidative energy metabolism in different cells (23,30–32). All these mechanisms may account for the decrease in the levels of ATP in melanoma cells induced by the local anesthetics (Fig. 4). A decrease in ATP is known to cause various cell abnormalities, since ATP is required for many energy-dependent systems (e.g., ion movement), as well as for various phosphorylation processes, and it

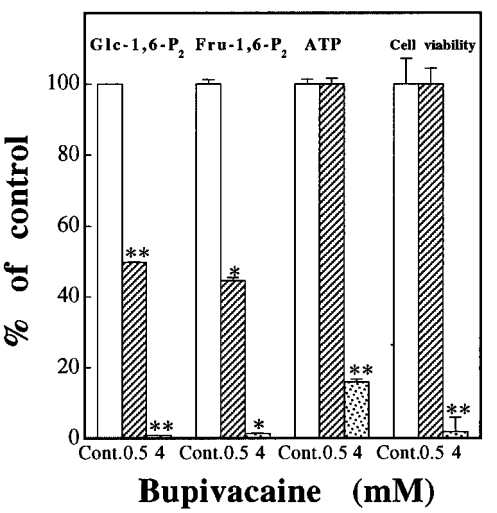

FIG. 6. Effect of bupivacaine on cell viability and its relation to the levels of ATP, glucose 1,6-bisphosphate (Glc-1,6-P_2), and fructose 1,6-bisphosphate (Fru-1,6-P_2). Cells were incubated with and without 0.5 and 4 mM bupivacaine for 20 min. 100% cell viability refers to 5×10^6 cells ml^{-1}; 100% ATP, glucose 1,6-bisphosphate, and fructose 1,6-bisphosphate levels refer to 44.75 ± 1.52, 0.366 ± 0.015, and 21.33 ± 1.20 (nmol/mg protein), respectively. Values are the means ± SEM of 2–3 separate experiments which were performed in triplicate $^{**}P < 0.005$, $^{*}P < 0.05$.

is well known that a fall in ATP eventually leads to cell death. This is expressed here by the dose-dependent reduction in the viability of B16 melanoma cells induced by the local anesthetics (Fig. 1), which correlated with the fall in ATP content (Figs. 4–6).

The decrease in the levels of the two allosteric signal molecules of glycolysis, glucose 1,6-bisphosphate and fructose 1,6-bisphosphate, induced by the local anesthetics, preceded the reduction in cell viability (Figs. 5 and 6). These results indicate that these changes are early events, and not a result of cell death.

Local anesthetics were reported to cause pathological accumulation of free intracellular Ca^{2+} (33), which is known to induce a reduction of cytosolic and cytoskeletal glycolysis, a fall in ATP, and cell death (9,10). Based on these and our recent reports (34,35), which have revealed that pathological accumulation of free intracellular Ca^{2+} mimicked the changes induced by local anesthetics reported here, it is possible that Ca^{2+} mediates the effects of these drugs.

In summary, the present results as well as the reports which have shown that local anesthetics inhibit cell proliferation (36) suggest that these drugs, and especially bupivacaine, are most promising agents for the treatment of melanoma.

ACKNOWLEDGMENTS

The skillful technical assistance of Mrs. H. Morgenstern and Mrs. H. Ben-Porat is acknowledged with thanks. This work was supported in part by the ALSAM Foundation (Los Angeles, CA), and the Health Sciences Research Center and the Research Authority of Bar-Ilan University (Ramat Gan, Israel).

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