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Short communication

Thermosensitizing Effects of Amiloride and 4,4-Diisothiocyanatostilbene-2,2'-disulfonic Acid on FsaII Mouse Fibrosarcoma

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Enhancement of the hyperthermia effect in FsaII fibrosarcoma of C3H mice *in vivo* by amiloride and 4,4-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was studied. Heating alone significantly increased the tumor lactic acid content and lowered the tumor energy levels, as indicated by the PCr and ATP contents which were measured using invasive chemical analysis. An i.p. injection of amiloride, DIDS, or amiloride combined with DIDS prior to heating further increased the lactic acid content and reduced the energy status in the tumors. Amiloride and DIDS may be useful in increasing the therapeutic efficacy of hyperthermia treatments by enhancing the reduction in tumor pH.

Keywords: Amiloride, DIDS, FsaII fibrosarcoma, Hyperthermia, Thermosensitizer.

Introduction

Hyperthermia in combination with radiotherapy and chemotherapy is being used for the control of human tumors. The efficacy of the hyperthermia as an adjuvant therapy is at least in part, attributed to the heat-induced tumor-vascular damage followed by the increase of acidity (Song *et al.*, 1980; Rhee *et al.*, 1982; 1984; Song, 1984; Lee *et al.*, 1986). Many attempts have been made to investigate the potency of drugs to lower intracellular pH and increase the thermosensitivity of tumors *in vivo*. It has been known that the intracellular pH (pH_i) is regulated mainly by the Na^+/H^+ antiport and Cl^-/HCO_3^- exchange through the cell membrane. Amiloride (3,5-diamino-6-chloro-*N*-(diami-nomethylene) pyrazine carboxamide) is a diuretic drug that blocks the Na^+/H^+ antiport and 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) is an inhibitor of Cl^-/HCO_3^- exchange (Roos and Boron, 1981; Vigne *et al.*, 1984). Lyons *et al.* (1992a; 1992b) reported that the amiloride and DIDS might increase the therapeutic efficacy of hyperthermia treatments by enhancing the reduction in tumor pH_i and the levels of the energy metabolites, ATP and phosphocreatine (PCr), in SCK mammary carcinomas of mice using non-invasive ^{31}P-NMR spectroscopy. However, the non-invasive ^{31}P-NMR spectroscopy has been known to have a noise problem. The invasive chemical analyses have not yet been conducted. In the present study, the effects of amiloride and DIDS on the contents of lactic acid and energy metabolites, ATP and PCr, in FsaII fibrosarcomas of mice were investigated. Particular attention was paid to determine the contents of lactic acid and the energy metabolites, ATP and PCr, in the tumors using invasive chemical methods.

Materials and Methods

Tumor The tumor used was the FsaII fibrosarcoma of C3H mice. Tumor cells in culture were harvested, trypsinized, and washed. About 5×10^4 cells in 0.05 ml culture medium were injected s.c. into the shaved hind legs of 10–12-wk old female C3H mice. The lactic acid, phosphocreatine, and ATP contents in the tumors after heating were measured when the tumors grew to 7–10 mm in diameter.

Hyperthermic treatment Animals were individually mounted and taped on specially designed mouse holders made of Plexiglass. The legs with tumors were protruded through 1.5-cm diameter holes in the holder and immobilized by anchoring a toe with thread to Plexiglass supporters which were attached adjacent to the holes. The animal holders were placed over a water bath and the legs were immersed into water preheated to 42.5 ± 0.02°C for 1 h. The tumor temperature, measured with a 29-gauge thermocouple, was about 0.3°C lower than the water temperature.

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For the sham-heating, the animals were mounted on the mouse holders, the legs with tumors were anchored to the leg supporters, and the animals were left at room temperature for 1 h.

Experimental groups The experimental groups consisted of treatment by amiloride injection, DIDS injection, amiloride combined with DIDS injection, sham-heating, heating at 42.5°C for 1 h, amiloride prior to heating, DIDS prior to heating, or amiloride combined with DIDS prior to heating. Each group consisted of seven C3H mice.

The animals were given amiloride or DIDS at a dose of 25 μg per g of body weight i.p. 1 h prior to heating. In the group of amiloride combined with DIDS prior to heating, the animals were given amiloride and DIDS i.p. 30 min apart prior to heating.

Amiloride and DIDS were dissolved on the day of the experiment in RPMI 1640 medium at the desired concentration and sterile-filtered.

Measurement of lactic acid content After heating, the animals were killed by cervical dislocation. The tumors were dissected, and immediately frozen in liquid nitrogen. The lactic acid content was measured using a procedure described by Gutmann and Wahlefeld (1977).

The frozen tissues were quickly weighed, and 10 vol of ice-cold 8% perchloric acid was immediately added. The tissues were homogenized with a glass-Teflon homogenizer. Following centrifugation at 4°C (10 min at 3000 × *g*), 0.2 ml supernatant was mixed with 2.8 ml reaction mixture (pH 9.2) containing 0.5 mM of hydrazine, 50 units of LDH (Sigma, Stock No. 826-6), 5 mg of NAD (Sigma, Stock No. 260-110), and 0.6 mM glycine in water. Following incubation in a 37°C water bath for 30 min, the NADH content was measured from the increase in spectroscopic extinction at 340 nm and the lactic acid content was calculated.

Measurement of phosphocreatine and ATP The frozen tissues were weighed quickly and then homogenized in 5 vol of ice-cold 10% perchloric acid with a glass-Teflon homogenizer. The homogenates were centrifuged at 4°C (10 min at 3000 × *g*), and 1.25 ml supernatant was neutralized with 20% potassium hydroxide in the presence of 2.5 μl universal indicator, and chilled in ice-water for 30 min. Following centrifugation at 4°C (10 min at 3000 × *g*), the supernatant was withdrawn and its phosphocreatine and ATP contents were measured.

Phosphocreatine Phosphocreatine was measured by determining creatine before ("free creatine") and after ("total creatine" = "free creatine" + "phosphocreatine") acid hydrolysis. The difference between these two determinations gives "phosphocreatine".

Creatine Creatine was measured by reaction with α-naphthol and diacetyl, which resulted in the formation of a colored complex detectable between 500 and 540 nm. A 200 μl sample of neutralized acid extract was added to 100 μl of α-naphthol made up in 10 ml of alkali solution, plus 100 μl of 0.05% diacetyl solution and 700 μl of distilled water. Upon completion of the reaction (approx. 15 min), absorbances were read at 520 nm against standards.

Total creatine (phosphocreatine plus creatine) A 200 μl sample of neutralized acid extract was added to 340 μl of distilled water, and the diluted sample was placed in a water bath at 65°C. After equilibration, 180 μl of 0.4 N HCl was added, and the contents were mixed and allowed to remain in the bath for 9 min. Then, 180 μl of 0.4 N NaOH was added and the mixtures were rapidly cooled to room temperature. One hundred μl of the α-naphthol made up in 10 ml of alkali solution and the same volume of 0.05% diacetyl solution were added to the acid-hydrolyzed sample. Upon completion of the reaction (approx. 15 min), absorbances were read at 520 nm against standards.

ATP The tissue extracts were analyzed for ATP by high-performance liquid chromatography using a Radial-Pak C18 (10-μm particles) reverse phase column. The solvent system consisted of HPLC grade acetonitrile-deionized distilled water (20:80, v/v) and 25 ml of Pic A (tetrabutylammonium phosphate) (Waters Corp., Milford, USA). The sample size was 10 μl per injection and the flow rate was 3 ml per min. The utilization of a fixed wavelength (254 nm) enabled quantification of ATP and calibrations were made by injection of a standard prior to analysis. Neutralized standards and samples were stable for at least 10 h at room temperature and indefinitely at −70°C (Rao *et al.*, 1982).

Results and Discussion

The lactic acid content in the control sham-heated FsaII fibrosarcoma was 9.8 μM per g of tumor. The lactic acid contents in the tumor injected with amiloride alone, DIDS alone, and amiloride combined with DIDS were 12.0 μM, 10.5 μM, and 13.0 μM, respectively, per g of tumor. The lactic acid content increased to 19.6 μM per g of tumor at the end of heating at 42.5°C for 1 h. The lactic acid content in the amiloride, DIDS, or amiloride combined with DIDS treatments prior to heating, rose significantly with a maximum increase of 35.2 μM per g of tumor for the latter group when compared with the heating-alone group (Fig. 1).

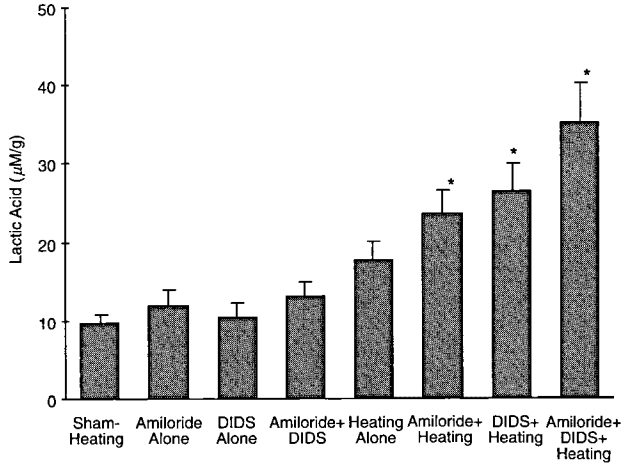

Fig. 1. Effect of hyperthermia on lactic acid content in FsaII fibrosarcoma of mice (mean ± S.D.). Seven measurements are shown. $*P < 0.05$ vs heated group.

The ATP content in the control sham-heated FsaII fibrosarcoma was 2.12 μM per g of tumor. The ATP contents per g of tumor in the groups injected with amiloride alone, DIDS alone, and amiloride combined with DIDS were 2.05 μM, 1.95 μM, and 1.90 μM, respectively. The ATP content decreased to 1.88 μM at the end of heating at 42.5°C for 1 h. The ATP content in the amiloride, DIDS, or amiloride combined with DIDS treatment prior to heating, profoundly decreased with a maximum depletion of 0.74 μM for the latter group when compared with the heating-alone group (Fig. 2).

The PCr content in the control sham-heated FsaII fibrosarcoma was 1.40 μM per g of tumor. That in the groups injected with amiloride alone, DIDS alone, and amiloride combined with DIDS, were 1.25 μM, 1.1 μM, and 1.05 μM, respectively, per g of tumor. It decreased to 0.96 μM per g at the end of heating at 42.5°C for 1 h. The PCr content in the amiloride, DIDS, or amiloride combined with DIDS treatment prior to heating, decreased profoundly with a maximum depletion of 0.37 μM per g for the latter group when compared with the heating-alone gorup (Fig. 3).

The present study demonstrated that heat induced an increase in the content of lactic acid in the FsaII fibrosarcoma of mice. Lee *et al.* (1986) reported that the lactic acid content in the SCK mammary carcinoma of A/J mice markedly increased when heated at 41.5°C or 43.5°C for 30 min. Dickson and Shah (1972) reported that anaerobic glycolysis is relatively thermoresistant as compared with the respiration process. It is conceivable that glycolysis increases in the heated tumors to compensate for the reduced respiration, resulting in an increased lactic acid formation. It should be pointed out, however, that not only the rate of formation but also the rate of removal of lactic acid by blood perfusion and diffusion would affect the lactic acid content in the tissues. When the blood perfusion in the tumors is still intact or even elevated during the earlier period of heating, the lactic acid formed may be removed promptly. However, the blood flow in the heated SCK tumor progressively deteriorates during and after heating (Kang and Song 1980; Song *et al.*, 1980; 1981) and removal of lactic acid may become sluggish. Furthermore, the deprivation of oxygen supply as a result of vascular damage in the heated tumors, compounded from the damaged respiration process, would inevitably increase the relative role of glycolysis and result in an increase in lactic acid content in the tumors (Lee *et al.*, 1986). Amiloride, DIDS, or amiloride combined with DIDS prior to heating profoundly increased the lactic acid content in the FsaII fibrosarcomas of C3H mice used in this study, indicating that the amiloride or the DIDS has a thermosensitizing effect, and the effect is additive with the combination of the amiloride and the DIDS.

In agreement with previous reports (Lilly *et al.*, 1984; Sijens *et al.*, 1989; Vaupel *et al.*, 1990; Lyons *et al.*, 1992a), we observed a profound reduction in high energy phosphate levels in the FsaII fibrosarcomas after heating. It should be mentioned that they used non-invasive ^{31}P-NMR spectroscopy which has been known to have a noise problem. An injection of 25 μg/g of amiloride, DIDS, or amiloride combined with DIDS to the tumor-bearing mice prior to heating, resulted in a further reduction of ATP and PCr levels compared with that detected after heating alone (Figs. 2 and 3).

It is unclear whether heating enhances the capability of amiloride or DIDS to reduce the ATP content or, conversely, whether amiloride or DIDS potentiated the ability of heat to reduce ATP content in the tumor cells. It is also not known whether the increased depletion of ATP

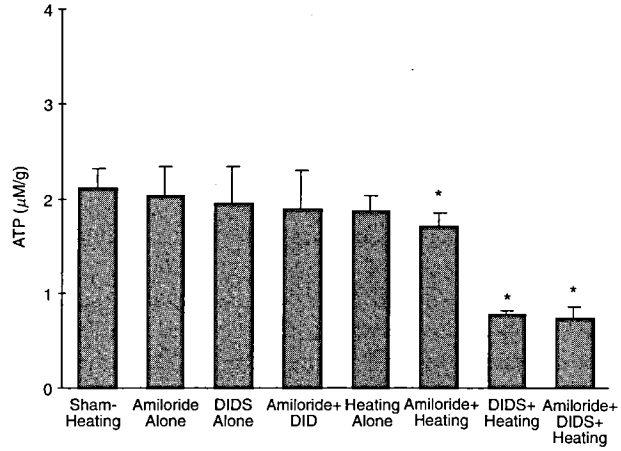

Fig. 2. Effect of hyperthermia treatment on ATP content in FsaII fibrosarcoma of mice (mean ± S.D.). Seven measurements are shown. $^*P < 0.05$ vs heated group.

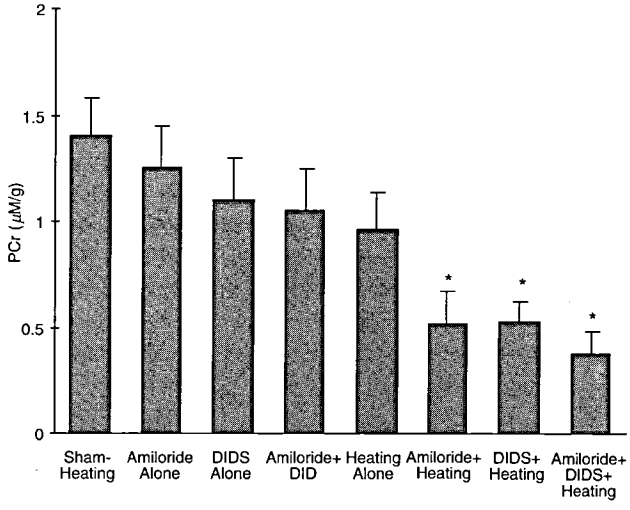

Fig. 3. Effect of hyperthermia treatment on PCr content in FsaII fibrosarcoma of mice (mean ± S.D.). Seven measurements are shown. $^*P < 0.05$ vs heated group.

observed is the result of direct drug interaction within the cell and whether it may just be a reflection of the greater degree of cell damage and death in the heated tumor in the presence of the drug (Lyons *et al.*, 1992a).

In agreement with the observation that amiloride and/or DIDS enhanced the heat-induced tumor growth delay, these drugs also enhanced the hyperthermia-induced reduction in the number of clonogenic cells in the tumors (Lyons *et al.*, 1992a). It has been reported that additional cell death occurs in FsaII tumors after heating due to the heat-induced noxious environment (Song *et al.*, 1980; Rhee *et al.*, 1982). Whether the drugs would increase the additional cell death that occurs after heating remains to be determined.

It is generally believed that the relative role of the Na^+/H^+ antiport in pH_i regulation in most cells is greater than that of Cl^-/HCO_3^- exchange. A plausible explanation is the increased intracellular lactic acid level due to the high rates of anaerobic glycolysis under hypoxic conditions. Decreased cellular energy levels under hypoxic conditions may also be involved. The antiport protein does not require ATP directly for its operation, but its function may be diminished under conditions of energy deprivation. The thermosensitization of tumors *in vivo* by amiloride and DIDS, in particular by a combination of these two drugs, may then be attributed to several mechanisms; namely, reduction in pH_i, reduction in ATP content, and the effect of heat-induced hypoxia in the tumors. The preliminary results of the experiments in our laboratory strongly indicate that tumors *in vivo* can be thermosensitized by amiloride alone or in combination with DIDS. The clinical usefulness of this drug as a thermosensitizer also remains to be investigated.

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