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L-carnitine prevents increase in diastolic [Ca^{2+}] induced by doxorubicin in cardiac cells

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Abstract

Doxorubicin is a highly effective anticancer chemotherapeutic agent that produces a dose-dependent cardiomyopathy that limits its clinical usefulness. We investigated the acute effects of doxorubicin on diastolic free Ca^{2+} concentrations ([Ca^{2+}]) and the cardioprotective action of L-carnitine in isolated cardiomyocytes loaded with fura-2/AM (acetoxymethyl ester). Exposure to 10^{-6}–10^{-4} M doxorubicin induced an elevation of diastolic calcium concentration ([Ca^{2+}]) that was concentration dependent. Nitrendipine failed to prevent the doxorubicin-induced elevation of [Ca^{2+}]. Incubation with L-carnitine (10^{-8} to 10^{-6} M) did not modify [Ca^{2+}]. Pretreatment of cardiomyocytes with L-carnitine 10^{-8}–10^{-7} M did not prevent the doxorubicin effect on [Ca^{2+}]. However, L-carnitine 10^{-6} M fully inhibited the increase in [Ca^{2+}] induced by this anthracycline derivative. These results indicate that acute exposure to doxorubicin impairs intracellular Ca^{2+} handling and that L-carnitine exerts a cardioprotective effect, in part by preventing the doxorubicin-induced increase in diastolic Ca^{2+} concentration.

Keywords: Ca^{2+}; Doxorubicin; L-Carnitine; Cardioprotection; Cardiotoxicity; Cardiomyocyte

1. Introduction

Doxorubicin is an anthracycline antibiotic widely used alone or as combination chemotherapy in the treatment of several types of cancer (Singal et al., 1987). Its clinical usefulness, however, is limited by its cardiotoxicity (Singal et al., 1987). Although the morphologic and clinical features of doxorubicin-induces cardiomyopathy are well characterized in humans as well as in a variety of research animals, the specific mechanism by which this antiblastic agent produces its cardiotoxicity is essentially unknown (Olson and Mushlin, 1990). Doxorubicin impairs several mechanisms important for cardiac function, including intracellular calcium homeostasis (Caroni et al., 1981; Salviati and Volpe, 1988; Vile and Winterbourn, 1990; Earm et al., 1994), which may play an important role in its cardiac toxicity (Jiang et al., 1994; Maeda et al., 1998).

A number of cardioprotective agents have been used to overcome the cardiotoxicity of doxorubicin with variable results (Maeda et al., 1997). One of them has been L-carnitine (β-hydroxy-γ-trimethylammonium butyric acid), a naturally occurring quaternary ammonium compound found in relatively high concentrations in cardiac tissue, which has been used to prevent or reverse the cardiotoxicity induced by doxorubicin (Neri et al., 1986; McFalss et al., 1986; Strauss et al., 1998).

In the present study, we examined the acute effects of doxorubicin on diastolic free calcium concentrations ([Ca^{2+}]) in isolated rat cardiomyocytes and demonstrated the cardioprotective effect of L-carnitine on doxorubicin-treated cells.

2. Materials and methods

2.1. Animals

The studies were performed with 13 male Sprague–Dawley rats, weighing 300 g, obtained from the Central Animal House of our Institute (IVIC). They were housed in a controlled environment and allowed free access to standard diet and water. The animal protocol was approved by the Institutional Care and Animal Use Committee fol-

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lowing the norms set out in the Guide for Care and Use of Laboratory Animals of National Institute of Health (NIH).

2.2. Diastolic $[Ca^{2+}]$ in single cardiomyocytes

Single cardiac myocytes from the ventricles of adult rats were dissociated according to a method previously described by Le Guennec et al. (1993). Isolated cardiomyocytes were subsequently loaded with 3 μM fura-2/AM dissolved in dimethyl sulfoxide (Molecular Probes, Eugene, OR, USA) at 37 °C for 30 min. Once the loading procedure was completed, the bathing solution was changed several times to remove the extracellular fura 2-AM. Fura-2-loaded cardiomyocytes were transferred to a temperature-regulated chamber (37 °C) mounted on the stage of an inverted epifluorescence microscope (Nikon, model Eclipse TE300, Nikon, Tokyo, Japan). During the experiments, cells were locally superfused with modified Tyrode's solution (37 °C). Excitation ultraviolet light wavelengths (340 and 380 nm) were selected with interference filters (Omega Optical, VT, USA) and a dichroic mirror, and the emitted light was filtered at 510 nm. Fluorescence signals obtained at 340 and 380 nm were measured with a fluorescence system interface and stored in a personal computer Pentium II, 333 MHz for data processing and analysis. The analysis was carried out with software video acquisition, version 4.3 (ION OPTIX, Milton, MA, USA). Diastolic Ca^{2+} concentration was estimated according to the method described by Tsien et al. (1982).

The modified Tyrode's solution had the same composition as normal Tyrode solution but NaCl (137 mM) was replaced by tetraethylammonium chloride 140 (mM). The pH was adjusted to 7.6. All drugs were prepared daily and diluted to the desired concentration in the modified Tyrode solution. These solutions were applied with a perfusion system, DAD-12 (ALA Scientific Instruments, Westbury, NY, USA), equipped with temperature regulation and pressure control. Doxorubicin was purchased form Sigma (St. Louis, USA) and L-carnitine was provided by Laboratorios Elmor (Caracas, Venezuela).

2.3. Statistics

Results are presented as means ± standard error of the means (S.E.M.). Statistical analysis was performed using Student's *t*-test for paired and unpaired observations; *n* represents the number of cardiomyocytes isolated from the 13 rats. Differences between means were regarded as statistically significant at $P < 0.05$.

3. Results

3.1. Effects of doxorubicin

The average diastolic Ca^{2+} concentration in quiescent cardiomyocytes was 105 ± 1.34 nM, $n = 16$. Fig. 1A shows

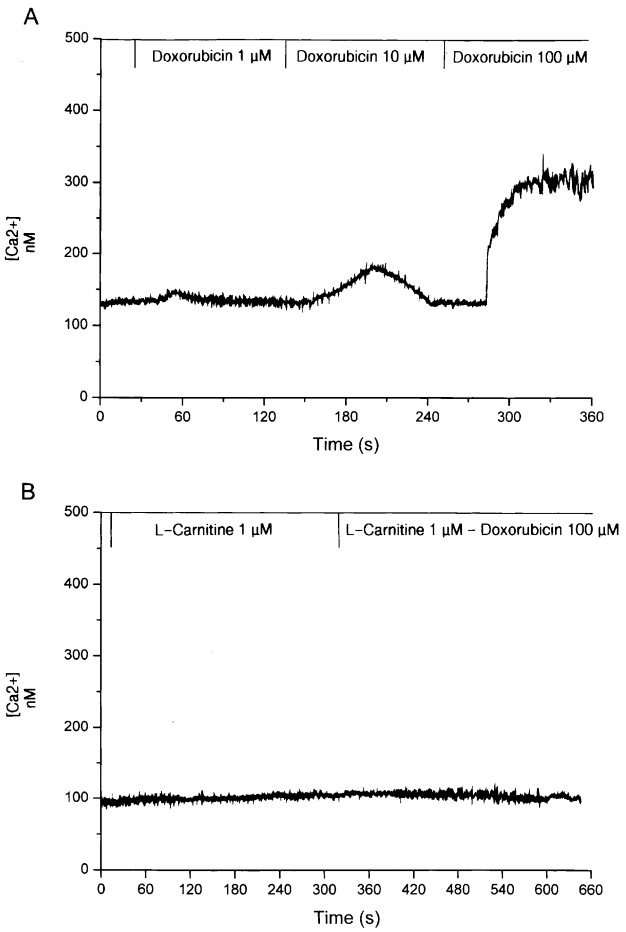

Fig. 1. (A) Doxorubicin-induced elevation of $[Ca^{2+}]$ in a single cardiomyocyte in a concentration-dependent manner. (B) L-carnitine did not modify diastolic $[Ca^{2+}]$ and prevented the doxorubicin-induced elevation of diastolic Ca^{2+} concentration.

that doxorubicin 1 μM did not induce a significant increase in diastolic calcium concentration (111 ± 2.12 nM, $n = 13$). However, doxorubicin at higher concentrations (10–100 μM) caused an increase in $[Ca^{2+}]$ that was dose dependent. Thus, doxorubicin 10 μM produced a slow elevation of $[Ca^{2+}]$ that reached a peak value of 181 ± 5.11 nM, $n = 12$. Thereafter, the diastolic calcium concentration subsided spontaneously, returning to the control concentration (Fig. 1A). Doxorubicin 100 μM produced a calcium release that was qualitatively and quantitatively different from that observed at lower concentrations. Doxorubicin induced a rapid increase in $[Ca^{2+}]$ (Fig. 1A) followed first by a plateau lasting several seconds (258 ± 16 s, $n = 9$) and then by a slow decline in the calcium signal that remained above the control level for at least 10 min after the doxorubicin solution was replaced by drug-free Tyrode solution (data not shown). In the presence of 100 μM doxorubicin, $[Ca^{2+}]$ reached a peak value of 318 ± 7.67 ($n = 13$). A summary of the experiments is presented in Table 1.

Table 1
Effects of doxorubicin on diastolic Ca^{2+} concentration

Experimental condition	$[Ca^{2+}]$	n
Control	105 ± 1.34	16
Doxorubicin 10^{-6} M	113 ± 1.11[a]	12
Doxorubicin 10^{-5} M	181 ± 5.11[b]	12
Doxorubicin 10^{-4} M	318 ± 7.67[b]	13
L-Carnitine 10^{-8} M	108 ± 1.29[a]	13
L-Carnitine 10^{-7} M	107 ± 1.59[a]	12
L-Carnitine 10^{-6} M	105 ± 1.32[a]	13
Nitrendipine 10^{-4} M	108 ± 1.11[a]	13
Doxorubicin 10^{-4} M + Nitrendipine 10^{-4} M	312 ± 7.31[b]	13
Doxorubicin 10^{-4} M + L-Carnitine 10^{-6} M	111 ± 1.17[a]	16
Caffeine 10^{-3} M	288 ± 9.59[b]	10

Ca^{2+} concentrations are expressed in nM (mean ± S.E.M). n represents the number of cardiomyocytes used under each experimental condition. They were isolated from five rats (control and doxorubicin experiments), three rats (L-carnitine experiments) and five rats (doxorubicin–nitrendipine, doxorubicin–L-carnitina and caffeine experiments).

[a] Not significant $P > 0.05$.

[b] Significant $P < 0.05$.

In addition, experiments were conducted in which cardiomyocytes were pretreated with 100 μM nitrendipine, a Ca^{2+} channel blocker agent, and then with 100 μM doxorubicin. Nitrendipine failed to prevent the Ca^{2+} overload observed upon exposure to doxorubicin (Table 1).

Our data demonstrate that acute administration of doxorubicin altered intracellular Ca^{2+} handling in quiescent isolated cardiomyocytes, inducing a significant elevation of diastolic Ca^{2+} concentration and that nitrendipine was not able to prevent the elevation of intracellular Ca^{2+} associated with doxorubicin treatment.

3.2. Cardioprotection by L-carnitine

The pharmacological interaction between L-carnitine and doxorubicin was studied to examine the cardioprotective effect of this amino acid in terms of preventing the doxorubicin-induced changes in intracellular calcium. Cardiomyocytes incubated with 10^{-8}–10^{-6} M L-carnitine did not show a significant change in $[Ca^{2+}]$ (Table 1). Pretreatment of cardiomyocytes with 0.01 and 0.1 μM L-carnitine did not prevent the elevation of $[Ca^{2+}]$ associated with the pharmacological effects of doxorubicin (data not showed). However, L-carnitine 1 μM blocked the doxorubicin-induced elevation of $[Ca^{2+}]$ at all the concentrations tested in this study (Fig. 1B). $[Ca^{2+}]$ measured in Tyrode solution was 107 ± 1.6 nM ($n = 17$), with 1 μM L-carnitine it was 108 ± 1.29 nM ($n = 15$) and with 1 μM L-carnitine plus 100 μM of doxorubicin it was 111 ± 1.17 nM ($n = 16$). In several experiments, once the preventive effects of L-carnitine on the doxorubicin-induced release of calcium were observed, the cardiomyocytes were exposed to 1 mM caffeine, to test the ability of the cell to release calcium in the presence of this drug. In these experiments $[Ca^{2+}]$ was elevated from 111 ± 1.17 nM ($n = 13$) to 288 ± 9.59 nM, $n = 10$ after caffeine treatment. These results demonstrated that the inhibition of calcium release in the presence of doxorubicin in cells pre-treated with L-carnitine appeared to be mediated by the pharmacological effect of this amino acid and was not related to cell injury. L-carnitine, at an appropriate concentration, was able to prevent the elevation of intracellular calcium induced by doxorubicin and this may explain in part the cardioprotection induced by this amino acid in doxorubicin-treated animals and patients (Neri et al., 1986; McFalss et al., 1986; Strauss et al., 1998).

4. Discussion

These results demonstrate for the first time that acute exposure to doxorubicin appears to be associated with an elevation of diastolic $[Ca^{2+}]$ in isolated cardiomyocytes and that L-carnitine, a natural amino acid present in cardiac cells, prevents the calcium elevation associated with doxorubicin.

Doxorubicin is a highly effective anticancer chemotherapeutic agent. It however produces a concentration-dependent cardiomyopathy that limits its clinical usefulness. The mechanism of doxorubicin cardiotoxicity is still uncertain but abnormal intracellular calcium handling has been implicated (Olson and Mushlin, 1990). Ca^{2+} signals modulate a large number of functions in cardiac cells, among others, contraction and relaxation processes. The signaling functions demand a very low concentration of Ca^{2+} ($\cong 100$ nM) inside cells, so that significant variations below and above this low concentration (physiological range) can induce profound changes in cardiac performance. The existence of calcium overload in doxorubicin-intoxicated myocardium was postulated a long time ago (Olson and Mushlin, 1990). However, this report constitutes the first direct demonstration that the diastolic Ca^{2+} concentration is increased upon acute exposure to doxorubicin. It is known that doxorubicin modifies several of the mechanisms that regulate intracellular Ca^{2+} concentrations in cardiac cells, which may end by increasing $[Ca^{2+}]$ (Caroni et al., 1981; Salviati and Volpe, 1988; Vile and Winterbourn, 1990; Earm et al., 1994). Diastolic calcium overload would induce random oscillations in $[Ca^{2+}]$ due to the spontaneous release of calcium from the sarcoplasmic reticulum, which in turn produce asynchronous mechanical activation that leads to an overall decline in myocardial force generation. Furthermore, diastolic Ca^{2+} overload may act as a trigger for a turbulent cascade of self-destructive processes, which involve activation of Ca^{2+}-activated neutral proteases.

Under our experimental conditions, the elevation of diastolic $[Ca^{2+}]$ induced by doxorubicin could be mediated either by its effects on the Ca^{2+}-ATPase (Vile and Winterbourn, 1990) or by its promotion of Ca^{2+} release from the sarcoplasmic reticulum (Salviati and Volpe, 1988) but not by its effects on Na^{+}/Ca^{2+} exchange (Caroni et al., 1981) since extracellular Na^{+} was removed from the extracellular solution and replaced by tetraethylammonium chloride. In addition, Ca^{2+} influx through Ca^{2+} channels (Earm et al., 1994) can also be ruled out since the data were collected from quiescent cardiac cells and nitrendipine failed to prevent Ca^{2+} overload. The nitrendipine finding supports the non-convincing results previously obtained in relation to the use of Ca^{2+} channel blockers against cardiac toxicity induced by doxorubicin (Maeda et al., 1998). Although the changes seen in diastolic Ca^{2+} concentration are not proven to be causal for doxorubicin-induced toxicity, this finding is consistent with the idea that Ca^{2+} overload is an important factor in cell death and leads to asynchronous mechanical activation and a decline in myocardial force generation.

L-carnitine is essential for the normal oxidation of fatty acids by the mitochondria and is involved in the transesterification and excretion of acyl-CoA esters, the oxidation of branched chain α-ketoacids, and the removal of potentially toxic acylcarnitine esters from the mitochondria. A number of studies have shown a protective effect of L-carnitine against doxorubicin-induced cardiotoxicity (Neri et al., 1986; McFalss et al., 1986; Strauss et al., 1998). The benefits of L-carnitine have been related to protection of mitochondrial structure, preservation of intracellular ATP levels, interference in the formation of the doxorubicin–cardiolipin complex, reduction in lipid peroxidation, improvement of cardiac energy metabolism and synthesis of shock proteins (Strauss et al., 1998). However, further experiments will be required to elucidate the exact mechanism by which L-carnitine exerts its cardioprotection in doxorubicin-treated cardiomyocytes.

In conclusion, direct measurement of $[Ca^{2+}]$ demonstrated that doxorubicin-induced cardiotoxicity appears to be associated with an intracellular buildup of diastolic Ca^{2+} concentration, which could generate a cascade of reactions ending with myoplasmic calcium overload and cell death. L-carnitine prevented the elevation of $[Ca^{2+}]$, which may explain in part the cardioprotective action of L-carnitine observed when it is administered to doxorubicin-treated patients and research animals.

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References

Caroni, P., Villani, F., Carafoli, E., 1981. The cardiotoxic antibiotic doxorubicin inhibits the Na^{+}/Ca^{2+} exchange of dog heart sarcolemmal vesicles. FEBS Lett. 130, 184–186.

Earm, Y.E., Ho, W.K., So, I., 1994. Effects of adriamycin on ionic currents in single cardiac myocytes of the rabbits. J. Mol. Cell Cardiol. 26, 163–172.

Jiang, J., Temma, K., Akera, T., 1994. Doxorubicin-induced changes in intracellular Ca^{2+} transients observed in cardiac myocytes isolated from guinea-pig heart. Can. J. Physiol. Pharmacol. 72, 622–631.

Le Guennec, J.Y., Peineau, N., Esnard, F., Lacampagne, A., Gannier, F., Argibay, J., Gauthier, F., Garnier, D., 1993. A simple method for calibrating collagenase/pronase E ratio to optimize heart cell isolation. Biol. Cell. 79, 161–165.

Maeda, A., Honda, M., Kuramochi, T., Tanaka, K., Takabatake, T., 1997. An angiotensin-corverting enzyme inhibitor protects against doxorubicin-induced impairment of calcium handling in neonatal rat cardiac myocytes. Clin. Exp. Pharmacol. Physiol. 24, 720–726.

Maeda, A., Honda, M., Kuramochi, T., Takabatake, T., 1998. Doxorubicin cardiotoxicity: diastolic cardiac myocytes dysfunction as a result of impaired calcium handling in isolated cardiac myocytes. Jpn. Circ. J. 62, 505–511.

McFalss, E.O., Paulson, D.J., Gilbert, E.F., Shug, A.L., 1986. Carnitine protection against adriamycin-induced cardiomyopathy in rats. Life Sci. 38, 497–505.

Neri, B., Cini-Beri, G., Bartalucci, S., Bandinelli, M., 1986. Protectitive effect of L-carnitine on cardiac metabolism damage induced by doxorubicin in vivo. Anticancer Res. 6, 659–662.

Olson, R.D., Mushlin, P.S., 1990. Doxorubicin cardiotoxicity: analysis of prevailing hypothesis. FASEB J. 4, 3076–3086.

Salviati, G., Volpe, P., 1988. Ca^{2+} release from sarcoplasmic reticulum of skinned fast and slow-twitch muscle fibers. Am. J. Physiol. 254, C459–C465.

Singal, P.K., Deally, C.M.R., Weinberg, L.E., 1987. Subcellular effects of adriamycin in the heart: a concise review. J. Mol. Cell Cardiol. 19, 817–828.

Strauss, M., Anselmi, G., Hermoso, T., Tejero, F., 1998. Carnitine promotes heat shock protein synthesis in adriamycin-induced cardiomyopathy in a neonatal rat experimental model. J. Mol. Cell Cardiol. 30, 2319–2325.

Tsien, R.Y., Pozzan, T., Rink, T., 1982. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. J. Cell Biol. 94, 325–334.

Vile, G., Winterbourn, C., 1990. Thiol oxidation and inhibition of Ca-ATPase by adriamycin in rabbit heart microsomes. Biochem. Pharmacol. 39, 769–774.