

## Bicarbonate exacerbates oxidative injury induced by antitumor antibiotic doxorubicin in cardiomyocytes

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Received 21 April 2000; accepted in final form 22 June 2000

**Konorev, Eugene A., Hao Zhang, Joy Joseph, M. Claire Kennedy, and B. Kalyanaraman.** Bicarbonate exacerbates oxidative injury induced by antitumor antibiotic doxorubicin in cardiomyocytes. *Am J Physiol Heart Circ Physiol* 279: H2424–H2430, 2000.—Doxorubicin, a broad-spectrum antitumor antibiotic, causes dose-dependent cardiomyopathy and heart failure. Although the exact molecular mechanisms of cardiotoxicity are not well established, oxidative mechanisms involving doxorubicin-induced superoxide anion production have been proposed. In this study, we show that bicarbonate, a physiologically relevant tissue component, greatly amplified doxorubicin-induced cardiomyocyte injury. Bicarbonate also enhanced inactivation of aconitase, a crucial tricarboxylic acid cycle enzyme, in cardiomyocytes exposed to doxorubicin. The cell-permeable superoxide dismutase mimetic, Mn(III)tetrakis (4-benzoic acid) porphyrin, reversed doxorubicin-induced cardiomyocyte injury. Bicarbonate enhanced the inactivation of purified mitochondrial aconitase in the xanthine/xanthine oxidase system, generating superoxide. The results suggest that bicarbonate amplifies the prooxidant effect of superoxide. Bicarbonate also caused an increased loading of cardiomyocytes with doxorubicin. We conclude that the bicarbonate-mediated increase in doxorubicin toxicity is due to increased intracellular loading of doxorubicin in cardiomyocytes and subsequent exacerbation of superoxide-mediated cardiomyocyte injury.

myocardium; aconitase; oxidative stress

THE ANTHRACYCLINE ANTIBIOTIC DOXORUBICIN (Dox) has been successfully used in cancer chemotherapy for decades (26). A major drawback of Dox treatment is the development of cardiomyopathy and heart failure, limiting the cumulative dose of the drug that can be administered to patients (24, 28). Several mechanisms for the development of cardiac complications induced by Dox have been advanced, including activation of protein kinases (29), apoptotic cascade in cardiomyocytes (27), and the suppression of muscle-specific gene expression (14, 16). It is generally agreed that Dox-induced generation of superoxide anion is partly responsible for the initiation of these secondary events (6, 27, 31). The use of superoxide anion scavengers and transfection of cardiac tissues with antioxidant pro-

teins [e.g., Mn-superoxide dismutase (SOD), metallothionein, catalase, etc.] counteracted Dox-mediated cardiomyocyte apoptosis and cell damage (17, 19, 32). These results further implicate a critical role of reactive oxygen species in the underlying mechanism(s) leading to apoptosis and the suppression of gene expression. However, antioxidant therapies for Dox-mediated cardiotoxicity have been only partially effective (22, 24). Clearly, more basic research is needed on factors that modulate oxidant formation at cellular and molecular levels. One of the key components in biological tissues and in cell culture experiments is bicarbonate anion ( $\text{HCO}_3^-$ ). However, very little attention has so far been paid to the modulatory role of  $\text{HCO}_3^-$  in xenobiotic cytotoxicity.  $\text{HCO}_3^-$  has been shown to dramatically alter oxidation and nitration reactions in biological systems (12, 21). In this study, we investigated the effect of  $\text{HCO}_3^-$  on oxidative cardiomyocyte injury induced by Dox.

$\text{HCO}_3^-$  is present at a high concentration (25 mM) in biological fluids and plays a major role in the regulation of pH in vivo, in cooperation with carbonic anhydrase and the  $\text{Na}^+$ -coupled and  $\text{Na}^+$ -independent  $\text{HCO}_3^-/\text{Cl}^-$  exchanges (13). In addition to regulating pH,  $\text{HCO}_3^-/\text{CO}_2$  has been recently shown to increase the reactivity of reactive nitrogen species (e.g., peroxynitrite) (21).  $\text{HCO}_3^-$  also enhanced the oxidation of luminol by reactive oxygen species, causing increased damage to critical biological targets and enzymes in oxidant-generating systems (20). Lysis of red blood cells was also enhanced in the presence of  $\text{HCO}_3^-$  (20). Despite its relevance to oxidative cell injury, the effect of  $\text{HCO}_3^-$  on cellular oxidative injury has not been thoroughly investigated.

We (19) have recently developed an adult rat cardiomyocyte model of Dox-induced myocardial injury. With the use of this model, we have shown that inactivation of aconitase parallels cardiomyocyte injury induced by Dox (19). Aconitase was proposed to be a sensitive marker for intracellular oxidant production (11, 25). Aconitase contains a  $[\text{4Fe-4S}]^{2+}$  cluster in its active center that is most sensitive to inactivation by

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superoxide. Aconitase is also rapidly inactivated by hydrogen peroxide in cultured cardiomyocytes, leading to the suppression of the tricarboxylic acid cycle, the principal source of high-energy phosphates produced from the oxidation of acetyl-coenzyme A in the heart muscle (15). Thus aconitase is a viable intracellular target for monitoring the effect of  $\text{HCO}_3^-$  on Dox-induced oxidative injury. In this paper, we report that  $\text{HCO}_3^-$  enhances the cardiomyocyte injury induced by Dox through an oxidative mechanism involving superoxide and aconitase and increased accumulation of Dox in cardiomyocytes.

## METHODS

### *Isolation and Culturing of Cardiomyocytes*

Adult rat ventricular cardiomyocytes were isolated from male Sprague-Dawley rats (175–225 g body wt) as previously described (19). Briefly, hearts were perfused with type II collagenase (200 U/ml; GIBCO-BRL) and minced to release cells from tissue chunks. The cell suspension was washed with buffer containing increasing concentrations of  $\text{CaCl}_2$ . To separate myocytes, the cell suspension was layered over a 4% BSA solution. Ventricular myocytes were then plated onto six-well plates or culture dishes with lids designed for optimal gas exchange. The culture medium was glutamine- and phenol red-free medium 199 with Earle's salts supplemented with 25 mM HEPES, 2 mg/ml BSA, 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 100 nM insulin, 100 IU/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. Intact cardiomyocytes adhered to the culture plates; damaged cells were washed away during the medium change 2 h after plating.

### *Experimental Time Course and Treatment Protocols*

After isolated cardiomyocytes were allowed to recover overnight, they were incubated at  $37^\circ\text{C}$  in the presence of indicated concentrations of Dox and 5%  $\text{CO}_2$  with or without  $\text{NaHCO}_3$  for 24–72 h. The toxicity of Dox to cardiomyocytes was assessed every 24 h using the release of the sarcosolic enzyme lactate dehydrogenase (LDH) into the culture medium. To measure the effect of intracellular accumulation of Dox on myocardial aconitase activity, cultured cardiomyocytes were incubated with 20  $\mu\text{M}$  Dox for 24 h. Under these experimental conditions, Dox-treated cells did not release LDH into the culture medium. In separate experiments, cardiomyocytes were treated with Dox in the presence of the cell-permeable SOD mimetic Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), the nitric oxide synthase inhibitor  $N^\omega$ -nitro-L-arginine methyl ester (L-NAME), the inhibitor of  $\text{Na}^+/\text{H}^+$  exchanger 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), or the inhibitor of  $\text{HCO}_3^-$ -dependent anion transporters DIDS. EIPA was dissolved in DMSO, and control cells were treated with vehicle alone.

### *Assessment of Cardiomyocyte Injury*

LDH was assayed in an aliquot of culture medium with the Optimized LDH reagent kit (Sigma Diagnostics) using the reaction of the reduction of pyruvate with an equimolar amount of NADH. The rate of decrease in absorbance at 340 nm was directly proportional to LDH activity in the sample.

### *Measurement of Dox Concentration in Cardiomyocytes*

Cardiomyocytes were washed twice with warm ( $37^\circ\text{C}$ ) PBS containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and lysed with a lysing buffer

containing 0.2% Triton X-100 and 100  $\mu\text{M}$  diethylenepentaacetic acid (DTPA) in PBS. Lysates were clarified by filtering with 0.2- $\mu\text{m}$  filters and analyzed using ultraviolet-visible spectroscopy at  $\lambda = 480$  nm. Concentrations of Dox were normalized to the protein content measured by the Bradford method (2).

### *Electron Spin Resonance Spin-Trapping*

The effect of  $\text{HCO}_3^-$  on formation of superoxide anion in the xanthine/xanthine oxidase system was monitored by electron spin resonance (ESR) spin-trapping (8, 23). Superoxide was detected using 2-ethoxycarbonyl-2-methyl-3,4-dihydro-3H-pyrrole-1-oxide (EMPO), a novel carboxylated analog of 5,5'-dimethyl-1-pyrroline N-oxide (DMPO). The EMPO-superoxide adduct is more persistent than the DMPO-superoxide. Spectra were recorded on a Varian E-109 spectrometer as previously described (10).

### *Assessment of Oxidative Injury*

**Measurement of aconitase activity.** Cardiomyocytes were washed twice with cold PBS and lysed with a lysing buffer containing 0.2% Triton X-100, 100  $\mu\text{M}$  DTPA, and 5 mM citrate in PBS. The activity of aconitase was measured in 100 mM Tris-HCl (pH 8.0) containing 20 mM DL-trisodium isocitrate. An extinction coefficient for cis-aconitate of  $3.6 \text{ mM}^{-1}$  at 240 nm was used. The presence of inactive  $[\text{3Fe-4S}]^+$  aconitase was determined by activating the cardiomyocyte lysates anaerobically in an anaerobic chamber in the presence of 5 mM dithiotreitol (DTT) and 0.5 mM ferrous ethylenediammonium sulfate. The difference in activity before and after DTT/ $\text{Fe}^{2+}$  treatment was a measure of the inactive  $[\text{3Fe-4S}]^+$  form of the enzyme.

Mitochondrial aconitase (2.3  $\mu\text{M}$ ), obtained and purified according to published methods (18), was incubated in 0.1 M of phosphate buffer (pH 7.5) containing 0.1 mM xanthine and 0.1 mM DTPA at  $25^\circ\text{C}$  in the presence and absence of 25 mM  $\text{HCO}_3^-$  and 2 mM of the aconitase substrate DL-isocitrate. Activity assays were performed before and after the addition of xanthine oxidase. The amount of added xanthine oxidase was determined by the activity needed to generate superoxide at a rate of 0.15  $\mu\text{M}/\text{min}$  in the absence of substrate and 1.5  $\mu\text{M}/\text{min}$  in the presence of isocitrate. The rate of superoxide production was determined by following the reduction of 50  $\mu\text{M}$  ferricytochrome c at 550 nm using an extinction coefficient of  $21 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ . The rate of superoxide production was unaffected by  $\text{HCO}_3^-$  (data not shown). All  $\text{HCO}_3^-$  buffers were stored in an atmosphere of 5%  $\text{CO}_2$ . The pH of the buffers was checked at the beginning and end of the incubation period. The increase in pH of  $\text{HCO}_3^-$ -containing buffers was always  $<0.15$  U. Inactivation of aconitase measured at pH 7.5 and pH 7.7 was identical.

**Intracellular oxidation of dichlorodihydrofluorescein.** Intracellular oxidant formation in the presence of Dox was assessed in cardiomyocytes loaded with 5- (and 6-) carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy- $\text{H}_2\text{DCFDA}$ ) (Molecular Probes), a nonfluorescent and cell-permeable analog that is converted into carboxy-2',7'-dichlorodihydrofluorescein after intracellular deacetylation and is oxidized to highly fluorescent carboxy-dichlorofluorescein (carboxy-DCF). The carboxylated form is retained more effectively inside the cells than the parent compound (7). In separate experiments, cultured cells were loaded with the oxidized cell-permeable analog, carboxy-dichlorofluorescein diacetate (carboxy-DCFDA; Molecular Probes), to determine whether incubations with Dox or  $\text{HCO}_3^-$  affect the transport and/or fluorescence of the compound inside the cells. Stock solutions

(10 mM) of carboxy- $\text{H}_2\text{DCFDA}$  and carboxy-DCFDA were prepared fresh in DMSO, kept on ice, and diluted to 10  $\mu\text{M}$  concentration before the experiment. Cardiomyocytes were washed with warm PBS containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . After cells were incubated for 15 min with 10  $\mu\text{M}$  fluorescent probes in PBS, they were washed and immediately analyzed using confocal microscopy.

**Confocal microscopy and image analysis.** Cells were examined using a Nikon Diaphot inverted microscope with the ODYSSEY confocal imaging system. Excitation of carboxy-DCF was achieved using a 488-nm line of a 300-mW argon ion laser. Rapid scanning of the observation field (2 s/scan) was used to minimize photooxidation artifacts. The fluorescence detection channel was equipped with a 515-nm long pass filter and a Hamamatsu R-928 photomultiplier. Images were acquired using Image 1/Metamorph software. The same acquisition parameters were used for all samples in the experiment. Quantitative image analysis was performed using SigmaScan Pro 5.0 software.

#### Statistical Analysis

All data are presented as means  $\pm$  SE. Data were subjected to one-way analysis of variance and then to the Student's *t*-test by using SigmaStat software (version 2.03, SPSS). Multiple group comparisons were performed using the Tukey test. Significance was accepted at the  $P < 0.05$  level.

## RESULTS

### Bicarbonate Enhances Cardiomyocyte Injury Induced by Dox

Cultured cardiomyocytes were incubated with Dox in HEPES-buffered or HEPES plus  $\text{HCO}_3^-$ -buffered media for 48 h. Dox (40  $\mu\text{M}$ ) had only a minimal effect on the release of LDH in the absence of  $\text{HCO}_3^-$ . However, Dox caused a significant concentration-dependent release of LDH from cardiomyocytes in the presence of  $\text{HCO}_3^-$  (Fig. 1A).  $\text{HCO}_3^-$  caused a concentration-dependent increase in Dox-induced toxicity (Fig. 1B). Incubation of cells in the presence of a cell-permeable SOD mimetic (MnTBAP) protected against Dox-induced injury. This suggests a role for superoxide or superoxide-derived species in cardiomyocyte injury (Fig. 1, C and D).  $\text{HCO}_3^-$  potentiates the toxic effects of Dox, which were also reversed by MnTBAP. On the other hand, the nitric oxide synthase inhibitor L-NAME (up to 7.5 mM) did not protect cardiomyocytes against Dox toxicity in the presence of  $\text{HCO}_3^-$  (data not shown).

### Bicarbonate Enhances Aconitase Inactivation by Dox

Dox caused rapid inactivation of aconitase in cardiomyocytes in the presence of  $\text{HCO}_3^-$ . The inhibition of aconitase was observed after 2 h of incubation with 20  $\mu\text{M}$  Dox; however, aconitase was only minimally inhibited in the absence of  $\text{HCO}_3^-$  (Fig. 2A). Figure 2B shows the reactivation of inactivated aconitase. Inactivated aconitase was reactivated by reduced thiols and  $\text{Fe}^{2+}$  after 2 h of incubation with Dox and  $\text{HCO}_3^-$ , indicating the recovery of the oxidized cluster. However, the enzyme could not be reactivated after 6-h incubation with Dox plus  $\text{HCO}_3^-$  (Fig. 2B).

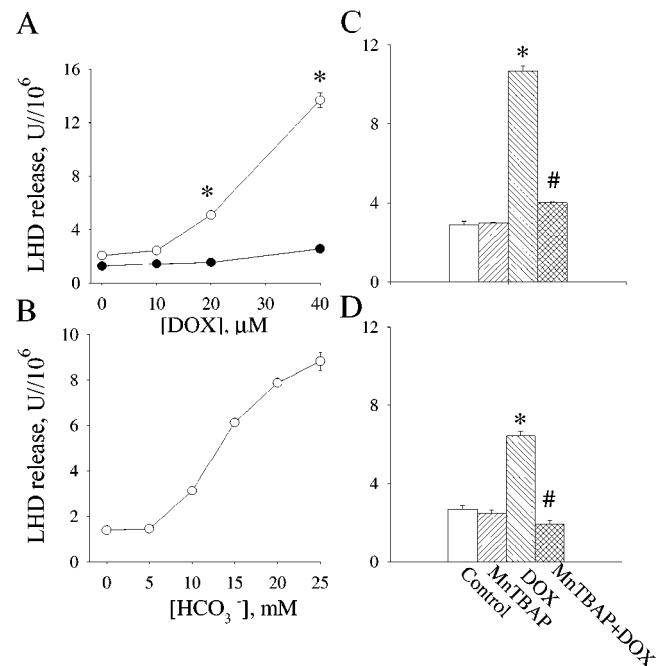


Fig. 1. Effect of bicarbonate ion ( $\text{HCO}_3^-$ ) and Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP) on cardiomyocyte injury induced by doxorubicin (Dox). Cardiomyocytes were plated onto 6-well plates at a density of  $6-8 \times 10^3$  cells/cm<sup>2</sup> and incubated with Dox. MnTBAP (200  $\mu\text{M}$ ) was present only in experiments shown in C and D, throughout the incubation period with Dox. An aliquot of medium was removed for lactate dehydrogenase (LDH) assay at the end of the incubation period. A: cardiomyocytes were incubated in HEPES (25 mM) plus  $\text{HCO}_3^-$  (25 mM)-buffered media (○) and HEPES-buffered media (●) in the presence of Dox for 48 h. \* $P < 0.05$  compared with HEPES-buffered media. B: cardiomyocytes were incubated with Dox (40  $\mu\text{M}$ ). C: cardiomyocytes were incubated with Dox (40  $\mu\text{M}$ ) in the presence of  $\text{HCO}_3^-$  (25 mM) for 48 h. D: cardiomyocytes were incubated with Dox (40  $\mu\text{M}$ ) in the absence of  $\text{HCO}_3^-$  for 72 h. For C and D, \* $P < 0.05$  compared with control and # $P < 0.05$  compared with Dox-treated cells. Results are presented as means  $\pm$  SE of 3 separate experiments, each performed in duplicate.

Superoxide was shown to oxidize the  $[4\text{Fe-4S}]^{2+}$  cluster of aconitase to yield the inactive  $[3\text{Fe-4S}]^{+}$  cluster (9). Therefore, we determined whether  $\text{HCO}_3^-$  enhances the inactivation of purified aconitase by superoxide. In the absence of substrate (i.e., isocitrate), aconitase was more susceptible to inactivation by superoxide produced by the xanthine/xanthine oxidase system.  $\text{HCO}_3^-$  did not affect the rate of inactivation by oxygen (Fig. 2C), but it accelerated the inhibition of aconitase induced by the superoxide-generating system. In the presence of isocitrate, the same trend was observed at a higher superoxide flux (Fig. 2D).

### Bicarbonate Enhances Formation of Intracellular Oxidants Induced by Dox

Intracellular oxidative stress was further assessed with the use of carboxy- $\text{H}_2\text{DCFDA}$ . Control myocytes incubated both in HEPES-buffered and HEPES plus  $\text{HCO}_3^-$ -buffered media showed minimal oxidative stress (Fig. 3, A and B). Dox in HEPES-buffered medium increased the formation of intracellular oxidants (Fig. 3C), and  $\text{HCO}_3^-$  enhanced the intracellular oxidative stress



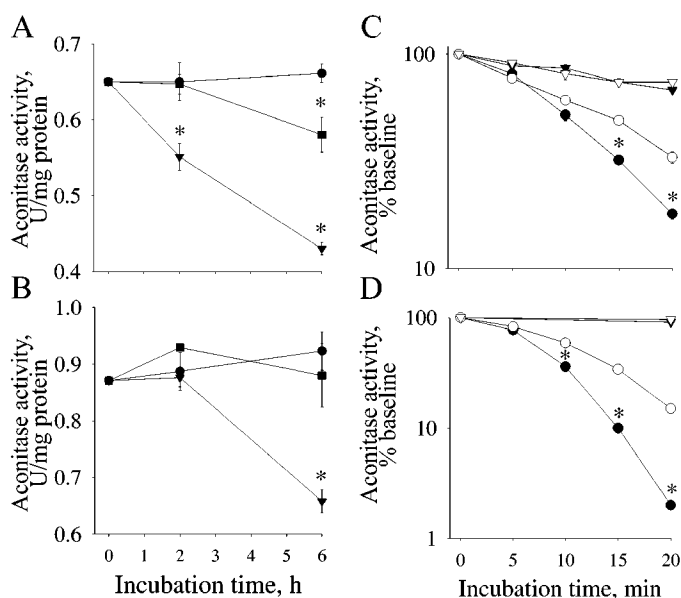


Fig. 2. Effect of  $\text{HCO}_3^-$  on the inactivation of aconitase induced by Dox and xanthine-xanthine oxidase. For A and B, cardiomyocytes were incubated with Dox (20  $\mu\text{M}$ ) for 20 h. There was no LDH release into the media throughout the incubation period with Dox under these conditions. Cells were washed and lysed at the end of the incubation period as described in METHODS. Aconitase activity was measured in the lysate after centrifugation. A: aconitase activity as a function of incubation time in Dox-free medium ( $\bullet$ ), in the presence of Dox ( $\blacksquare$ ), and in the presence of Dox and  $\text{HCO}_3^-$  ( $\blacktriangle$ ). B: reactivated aconitase was measured using  $\text{Fe}^{2+}$  in the presence of dithiothreitol (DTT) as described in METHODS. Symbols indicate the same experimental conditions as in A.  $*P < 0.05$  compared with the Dox-free group. Results are presented as means  $\pm$  SE of 3 separate experiments, each performed in duplicate. C and D: inactivation of purified aconitase by a superoxide anion-generating system. Purified mitochondrial aconitase (2.3  $\mu\text{M}$ ) was incubated at 25°C in 0.1 M phosphate buffer (pH 7.5) in the presence or absence of xanthine oxidase. Aliquots were removed, and activities were determined at the indicated times. C: aconitase incubated in the absence of substrate in control medium ( $\Delta$ ), with  $\text{HCO}_3^-$  (25 mM) ( $\blacktriangle$ ), with xanthine oxidase ( $\circ$ ), and with xanthine oxidase and  $\text{HCO}_3^-$  ( $\bullet$ ). Superoxide was generated at a rate of 0.15  $\mu\text{M}/\text{min}$ . D: aconitase incubated with trisodium isocitrate as a substrate; superoxide was generated at a rate of 1.5  $\mu\text{M}/\text{min}$ . Symbols indicate the same experimental conditions as in C.  $*P < 0.05$  compared with xanthine oxidase treatment in  $\text{HCO}_3^-$ -free buffer. Results are presented as means  $\pm$  SE of 2 separate experiments, each performed in triplicate.

induced by Dox (Fig. 3D). The quantitative analysis of cell fluorescence intensity is shown in Fig. 3F. To substantiate that the differences in intracellular fluorescence are primarily due to increased oxidation and not to differences in intracellular pH, loading of the probe, or its leakage, cardiomyocytes were loaded with the oxidized analog, carboxy-DCFDA. A moderate reduction in the fluorescence intensity of carboxy-DCF was observed in cardiomyocytes incubated with Dox and  $\text{HCO}_3^-$  (Fig. 3E), and this can only lead to an underestimation of intracellular oxidative stress in cardiomyocytes treated with Dox in the presence of  $\text{HCO}_3^-$ .

#### Effect of Bicarbonate on Superoxide Adduct Formation

Superoxide generated in the xanthine/xanthine oxidase system was trapped by the spin-trap EMPO (23).

EMPO has been reported to trap superoxide anion, forming a more persistent EMPO-superoxide adduct (EMPO-OOH) (23). Unlike DMPO-OOH, EMPO-OOH does not spontaneously decay to the corresponding EMPO-hydroxyl adduct (EMPO-OH). The half-life of the EMPO-OOH adduct is about eight times longer than that reported for DMPO-OOH (23). In the presence of 25 mM  $\text{HCO}_3^-$ , the steady-state concentration of the EMPO-OOH adduct was reduced by 50% (Fig. 4, A and B).  $\text{HCO}_3^-$ -mediated inhibition of the EMPO-OOH adduct formation is tentatively attributed to a slower reaction between superoxide and  $\text{HCO}_3^-$ , producing another oxidant (Fig. 4, C and D). Results from these spin-trapping studies indicate that superoxide can react with  $\text{HCO}_3^-$ .

#### Bicarbonate Enhances Accumulation of Dox in Cardiomyocytes

We tested the hypothesis that  $\text{HCO}_3^-$  increases the accumulation of Dox in cardiomyocytes, leading to enhanced oxidative stress and cardiomyocyte injury. Figure 5A shows the time-dependent accumulation of Dox in cardiomyocytes. Cardiomyocytes accumulated Dox more rapidly in the presence of  $\text{HCO}_3^-$ , so that the concentration of Dox increased more than threefold after 24-h incubation. Accumulation of Dox in cardiomyocytes during a 24-h incubation increased with increasing concentrations of  $\text{HCO}_3^-$  (Fig. 5B). Because  $\text{HCO}_3^-$  is known to moderately elevate intracellular pH

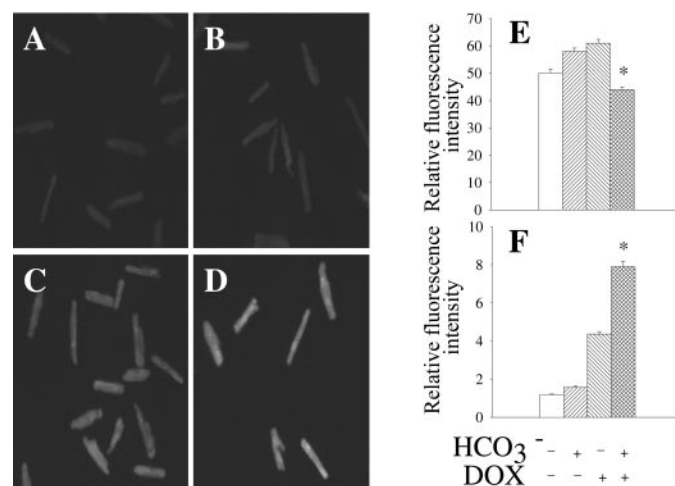


Fig. 3. Effect of  $\text{HCO}_3^-$  on intracellular oxidative stress induced by Dox in cardiomyocytes. Cardiomyocytes were plated onto 35-mm dishes and incubated with 40  $\mu\text{M}$  Dox for 3 h. Cells were washed and incubated with 10  $\mu\text{M}$  dichlorodihydrofluorescein diacetate (carboxy- $\text{H}_2\text{DCFDA}$ ; A–D and F) or carboxy-dichlorofluorescein diacetate (carboxy-DCFDA; E) for 15 min at room temperature in the dark. Cells were washed and immediately studied using confocal microscopy as described in METHODS. Confocal images were quantitatively analyzed for fluorescence intensity. A: control, no  $\text{HCO}_3^-$ . B: control, 25 mM  $\text{HCO}_3^-$ . C: Dox, no  $\text{HCO}_3^-$ . D: Dox, 25 mM  $\text{HCO}_3^-$ . E: analysis of fluorescence intensity of cardiomyocytes incubated with carboxy-DCFDA (oxidized, fluorescent form). F: summarized data for the intracellular oxidation of carboxy- $\text{H}_2\text{DCFDA}$  (reduced, nonfluorescent form); 30–50 cells were counted per group.  $*P < 0.05$  compared with Dox-treated cells in the absence of  $\text{HCO}_3^-$ . Results are presented as means  $\pm$  SE.

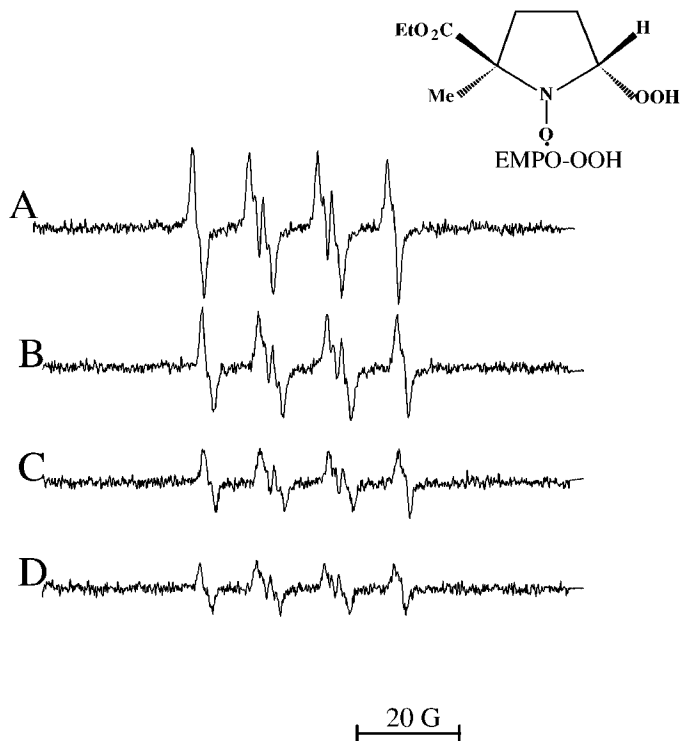


Fig. 4. Effect of  $\text{HCO}_3^-$  on the formation of 2-ethoxycarbonyl-2-methyl-3,4-dihydro-3H-pyrrole-1-oxide (EMPO)-superoxide adduct (inset). EMPO was incubated with 0.05 U/ml xanthine oxidase and 1 mM xanthine in phosphate buffer (100 mM, pH 7.5) containing 100  $\mu\text{M}$  diethylenepentaacetic acid at room temperature for 15 min. A: EMPO (25 mM). B: EMPO (25 mM) in the presence of  $\text{HCO}_3^-$  (25 mM). C: EMPO (5 mM). D: EMPO (5 mM) in the presence of  $\text{HCO}_3^-$  (25 mM). Electron spin resonance spectra were recorded at room temperature. Spectrometer equipped with a  $\text{TE}_{102}$  cavity was operated at 9.5 GHz with 100 kHz field modulation. Other conditions were the following: modulation amplitude, 1 G; time constant, 64 ms; scan time, 2 min; and microwave power, 5 mW.

in cardiomyocytes (3), we determined whether intracellular modification caused by inhibitors of  $\text{H}^+$  efflux would prevent the increased accumulation of Dox in cardiomyocytes. We used DIDS, an inhibitor of  $\text{HCO}_3^-/\text{Cl}^-$  exchangers, and EIPA, a specific inhibitor of the  $\text{Na}^+/\text{H}^+$  exchanger. These pH-regulating mechanisms are known to be present in cardiomyocytes, and their inhibitors have been shown to significantly lower intracellular pH in these cells (3, 30). Neither DIDS nor EIPA alone, nor their combined administration, changed the amount of Dox accumulated in cardiomyocytes in the presence of  $\text{HCO}_3^-$  (data not shown).

#### Effect of Extracellular pH on Accumulation of Dox in Cardiomyocytes

The changes in extracellular pH, induced by  $\text{HCO}_3^-$ , could affect the polarity of the Dox molecule and facilitate its transport through the lipid bilayer. In the following experiments, we tested whether 1)  $\text{HCO}_3^-$  could change the pH of the culture media during the incubation of cardiomyocytes in 5%  $\text{CO}_2$  and subsequently affect the accumulation of Dox in cardiomyocytes and 2) increased incorporation of intracellular

Dox would affect cardiomyocyte injury by an oxidative mechanism. We tested the time-dependent pH changes in HEPES-buffered or HEPES plus  $\text{HCO}_3^-$ -buffered media incubated in an atmosphere of 5%  $\text{CO}_2$ -95% air at 37°C. The pH level was adjusted to 7.1, and, after vacuum filtration and storage, media were readjusted to 37°C for cell plating. After the media were incubated in a  $\text{CO}_2$  incubator for 1 h, the pH level in the HEPES-buffered medium dropped to  $\sim 6.6$  and remained constant thereafter. In contrast, the pH initially rose to 7.5 in the presence of  $\text{HCO}_3^-$  and then slowly decreased to 7.4 after 24 h of incubation. In an experiment represented in Fig. 5C, the pH of the culture media was adjusted to values from 6.2 to 7.1 (in the presence of  $\text{HCO}_3^-$ ) or from 7.1 to 8.0 (in the absence of  $\text{HCO}_3^-$ ) at the beginning of incubation with Dox. The elevation of extracellular pH measured at the end of the 24-h incubation period increased the accumulation of Dox in both HEPES-buffered and HEPES plus  $\text{HCO}_3^-$ -buffered media (Fig. 5C). The levels of intracellular Dox, as

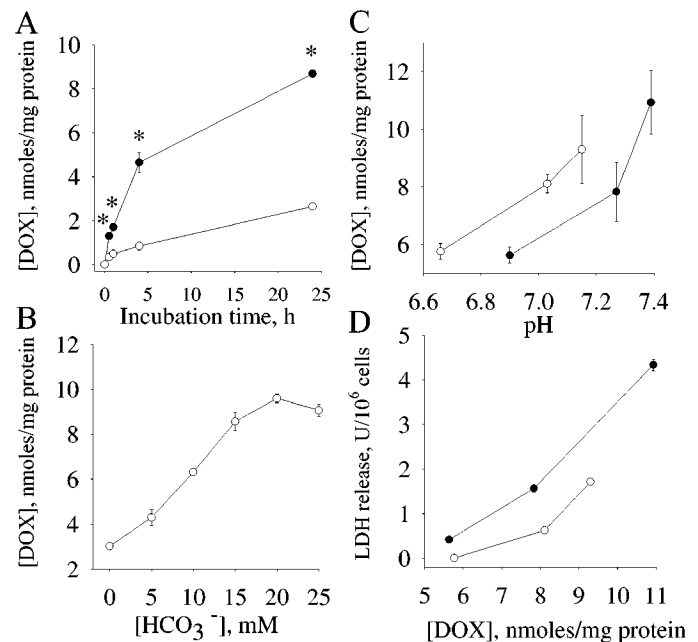


Fig. 5. Effect of changes in extracellular pH on the accumulation and toxicity of Dox in cardiomyocytes. Cardiomyocytes were plated onto 100-mm dishes, incubated with Dox, washed, and lysed at the end of the incubation period, as described in METHODS, to release entrapped Dox.  $\circ$ , No  $\text{HCO}_3^-$ ;  $\bullet$ , 25 mM  $\text{HCO}_3^-$ . A: cardiomyocytes were incubated with Dox (20  $\mu\text{M}$ ). \* $P < 0.05$  compared with  $\text{HCO}_3^-$ -free media. B: cardiomyocytes were incubated with Dox (20  $\mu\text{M}$ ) for 24 h. C: pH of the culture media was adjusted to values from 6.2 to 7.1 (in the presence of  $\text{HCO}_3^-$ ) or from 7.1 to 8.0 (in the absence of  $\text{HCO}_3^-$ ) at the beginning of the incubation period with Dox (20  $\mu\text{M}$ ). After we incubated the media for a 24-h incubation period in a  $\text{CO}_2$  incubator, the pH of the media was measured, the cells were washed and lysed, and the concentration of Dox was assayed as described in METHODS. D: LDH release from cardiomyocytes as a function of the intracellular concentration of Dox. The pH of the culture media was adjusted as described in C. Cardiomyocytes were incubated with Dox (30  $\mu\text{M}$ ) for 48 h. An aliquot of the medium was taken at the end of the incubation period for LDH assay. Cardiomyocytes were washed and lysed, and the concentration of Dox was measured as described in METHODS. Results are presented as means  $\pm$  SE of 3 separate experiments, each performed in duplicate.

affected by changes in extracellular pH, were plotted against elevations in LDH release obtained under the same experimental conditions (Fig. 5D). Higher levels of Dox resulted in increased LDH release from cardiomyocytes.

## DISCUSSION

In this study, we discovered that  $\text{HCO}_3^-$  exacerbated superoxide-mediated inactivation of purified aconitase, causing irreversible inactivation of aconitase in cardiomyocytes induced by Dox.  $\text{HCO}_3^-$  also increased the loading of cardiomyocytes with Dox. These results led us to suggest that several factors may contribute to the enhancement of Dox-induced cardiomyocyte injury by  $\text{HCO}_3^-$ .

### *Bicarbonate Enhances Dox-Induced Cardiomyocyte Injury Via an Oxidative Mechanism*

$\text{HCO}_3^-$  generates substantial amounts of  $\text{CO}_2$  in aqueous solutions that can react with peroxyxynitrite anion, the product of the reaction of superoxide with nitric oxide. The intermediate of this reaction,  $\text{ONOOCO}_2^-$ , was shown to exhibit enhanced nitrating and oxidizing properties (21). However, cardiomyocyte injury induced by Dox in the presence of  $\text{HCO}_3^-$  was not prevented by the nitric oxide synthase inhibitor L-NAME, suggesting that peroxyxynitrite is probably not responsible for  $\text{HCO}_3^-$ -enhanced injury. An earlier report (20) has suggested that oxidation of luminol in oxidant-generating systems is entirely dependent on the presence of  $\text{HCO}_3^-$ . The reactions of  $\text{HCO}_3^-$  with superoxide anion and hydroxyl radical have been suggested to form the carbonate anion radical (12, 20). The carbonate anion radical has been shown to rapidly oxidize sulfur-containing amino acids (rate constant  $k = 10^6\text{--}10^7 \text{ M}^{-1}\cdot\text{s}^{-1}$ ) and aromatic compounds, such as indol and its derivatives ( $k > 10^8 \text{ M}^{-1}\cdot\text{s}^{-1}$ ) (4). In this report, we show that  $\text{HCO}_3^-$  increases the rate of inactivation of purified aconitase by a superoxide-generating system. In cultured cardiomyocytes, the rate of inactivation of aconitase by Dox was increased in the presence of  $\text{HCO}_3^-$  compared with  $\text{HCO}_3^-$ -free media. The inactivation of aconitase by Dox was presumably due to the formation of a  $[\text{3Fe-4S}]^+$  cluster in the initial phase of incubation, because it could be reactivated by  $\text{Fe}^{2+}$  in the presence of reduced thiols. This reversible mechanism of oxidation is attributed to the release of the solvent-exposed  $\text{Fe}^{2+}$  atom during the oxidation of the cluster by superoxide. Prolonged incubation, however, resulted in the irreversible inhibition of aconitase. These results suggest a different mechanism of inactivation, which could be either destruction and/or loss of the cluster, or the oxidative modification of apoprotein. The clusterless cytosolic aconitase effectively functions as an iron regulatory protein 1. Iron regulatory protein 1 induces changes in cellular iron metabolism that lead to the increased availability of iron in cells. Elevated intracellular iron is likely to provoke the increased oxidative cell damage induced by reactive oxygen species. It may play a role in the

mechanism of Dox-induced cardiomyocyte injury and, specifically, in the development of Dox-induced apoptosis of cardiomyocytes (27).

### *Bicarbonate Enhances Intracellular Accumulation of Dox*

$\text{HCO}_3^-$  increases the net proton efflux from cardiomyocytes via membrane-based anion transporters, contributing to the recovery from intracellular acidosis (30).  $\text{HCO}_3^-$  moderately increases intracellular pH in intact cardiac tissue (3). The  $\text{HCO}_3^-$ -dependent rise in intracellular pH is blocked by DIDS (30). DIDS did not affect the intracellular concentration of Dox or cardiomyocyte injury, indicating that the enhanced cardiomyocyte injury in the presence of  $\text{HCO}_3^-$  was unlikely to be due to the changes in intracellular pH.  $\text{HCO}_3^-$  serves as a buffering system that maintains pH in extracellular fluids. The transport of Dox occurs mostly by diffusion of uncharged molecules through the lipid domain of the cell membrane (5). The acidic dissociation constant of the  $\text{NH}_3^+$  group of the Dox molecule has been reported to be 7.6 at  $37^\circ\text{C}$  (5). A major portion of Dox is protonated at lower pH, leading to reduced transport through the lipid bilayer and decreased cardiomyocyte injury. Increasing the pH facilitates the transport of the unprotonated Dox into cardiomyocytes. The transport of Dox in red blood cells was shown to increase with increasing pH (5). The rate of Dox uptake into the Chinese hamster cells was reported to increase with the elevation of pH of the culture medium (1). Therefore, the exacerbated oxidative stress and cardiomyocyte injury induced by Dox in the presence of  $\text{HCO}_3^-$  may be partly due to the increased loading of the drug into cardiomyocytes.

In summary, the cardiomyocyte injury induced by Dox is augmented by  $\text{HCO}_3^-$ . This is attributed to either increased loading of cardiomyocytes with the drug or to enhanced oxidative damage by  $\text{HCO}_3^-$  or to both.

This work was supported by National Institutes of Health Grants CA-77822 (to B. Kalyanaraman) and RR-01008 and GM-51831 (to M. C. Kennedy).

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