Enhanced Expression of Superoxide Dismutase Messenger RNA in Viral Myocarditis

An SH-dependent Reduction of its Expression and Myocardial Injury

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Abstract

The oxygen free radical system has been reported to be activated by influenza virus infection in the lungs. However, the involvement of oxygen radicals in viral myocarditis is still unknown. Captopril, an angiotensin–converting enzyme (ACE) inhibitor and potent free radical scavenger with a sulfhydryl group, was effective for the treatment of viral myocarditis, while enalapril, an ACE inhibitor without a sulfhydryl group, was not effective against acute myocarditis. In this study, we investigated the role of oxygen radicals in the pathogenesis of viral myocarditis and the therapeutic effects of agents with a sulfhydryl group.

4-wk-old BALB/c mice were inoculated with the encephalomyocarditis virus, and treated with captopril or *N*,2-mercapto-propionyl glycine (MPG), a sulfhydryl-containing amino acid derivative without ACE inhibiting property, from days 4 to 14. On day 14, captopril and MPG significantly improved survival of mice and myocardial injury (necrosis, cellular infiltration, and calcification) in a dose-dependent manner compared with the infected control group. Thus, captopril and MPG were effective for the treatment of virus-induced myocarditis. Furthermore, a striking induction of manganese superoxide dismutase (Mn-SOD) and copper/zinc SOD (Cu/Zn-SOD) mRNAs in infected hearts was found (8–13-fold for Mn-SOD and 4–11-fold for Cu/Zn-SOD) when compared with age-matched uninfected mice hearts. MPG completely inhibited the increase of both mRNAs, even when treatment was started on day 4.

Thus, oxygen radicals may play an important role in the pathogenesis of viral myocarditis, and a therapeutic approach by eliminating oxygen radicals seems possible. (*J. Clin. Invest.* 1993. 91:2727–2733.) Key words: captopril • superoxide dismutase • messenger RNA • myocarditis • free radicals

Introduction

Acute myocarditis is generally considered to be a benign condition from which most patients recover completely. However, a small but significant number of patients suffer from residual myocardial abnormalities, and some of them eventually progress to dilated cardiomyopathy (1–3). Therefore, the clinical management of acute myocarditis is important to prevent this progression to dilated cardiomyopathy. Although immunomodulating (4–5) and antiviral (6) therapy has proved to be effective for experimental viral myocarditis, treatment must be started at a very early stage. Alternative treatment, which can be started at a later stage of the disease, is expected for viral myocarditis.

Recently captopril, an angiotensin–converting enzyme (ACE)[1] inhibitor with a sulfhydryl group, was shown to reduce myocardial injury and congestive heart failure in virus-induced murine myocarditis, even when treatment was started on day 4 after the viral inoculation (7–9), but the mechanism of action is still not clear. We subsequently showed that enalapril, an ACE inhibitor without a sulfhydryl group, had no significant effect on myocardial injury, although it improved congestive heart failure in the same model (10). This suggested that the beneficial effect of captopril on viral myocarditis might depend not on its ACE inhibition, but on its possession of a sulfhydryl group.

Pathological damage caused by oxygen radicals generated in the xanthine-xanthine oxidase system, has been reported in influenza virus–infected lungs (11). However, the involvement of oxygen radicals in viral myocarditis is not known. Reactive oxygen radicals can oxidize membrane lipids, cellular proteins, and nucleic acids, resulting in cell damage or death (12). It is well known that oxygen radicals are important mediators of myocardial ischemia/reperfusion injury (13–15) against which sulfhydryl compounds have been shown to give protection by scavenging the oxygen radicals (16–21). It is possible that captopril improved the virus-induced myocardial injury in our model by acting as a free radical scavenger.

Our current studies were designed to find out if a sulfhydryl group can play a role in the treatment of encephalomyocarditis virus–induced myocarditis by comparing the effects of captopril and *N*,2-mercapto-propionyl glycine (MPG), a sulfhydryl-containing amino acid derivative without ACE-inhibiting property, and whether the sulfhydryl groups act as free radical scavengers.

We demonstrated that captopril and MPG improved the survival of mice and myocardial injury such as cellular infiltration, necrosis, and calcification, as well as congestive heart failure caused by viral infection. The effects of MPG were similar to those of captopril in our murine myocarditis model. A striking increase of manganese superoxide dismutase (Mn-SOD)

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1. *Abbreviations used in this paper:* ACE, angiotensin–converting enzyme; Cu/Zn-SOD, copper/zinc SOD; Mn-SOD, manganese superoxide dismutase; MPG, *N*,2-mercapto-propionyl glycine.

and copper/zinc SOD (Cu/Zn-SOD) mRNAs was detected in the hearts of the mice inoculated with encephalomyocarditis virus. Also, MPG completely inhibited the increase of both mRNAs, even when treatment was started on day 4 after the viral inoculation. It appears that sulfhydryl compounds can reduce virus-induced myocardial injury by eliminating oxygen radicals.

This is the first report to suggest the participation of oxygen radicals in the pathogenesis of virus-induced myocarditis. A therapeutic approach by eliminating oxygen radicals seems possible.

Methods

Animal treatment. Inbred BALB/c mice were obtained from the Shizuoka Agricultural Cooperative Association (Shizuoka, Japan) at 4 wk of age. The mice were inoculated intraperitoneally with a myocardiotrophic variant of encephalomyocarditis virus suspension containing 100 plaque-forming units. On day 4, the surviving mice (captopril experiment: $n = 108$; MPG experiment: $n = 110$) were randomized and treated orally with captopril, 10 mg/kg ($n = 24$), 30 mg/kg ($n = 21$), 100 mg/kg ($n = 26$), or intraperitoneally with MPG, 8 mg/kg ($n = 30$), 25 mg/kg ($n = 25$), 75 mg/kg ($n = 25$) once a day from days 4 to 14 after the viral inoculation. The infected control mice (placebo group; captopril experiment: $n = 37$, MPG experiment: $n = 30$) were given 0.1 ml of a glucose solution orally or intraperitoneally. As age-matched controls, uninfected mice either treated with drugs (uninfected treated group: $n = 10$) or given no treatment (uninfected normal group: $n = 10$) were processed according to the same protocol.

On day 14, the surviving mice were killed. The body weight and heart weight were measured and hearts were prepared for histological examination.

For Northern blot analysis, the hearts of age-matched uninfected mice, infected mice, and infected mice treated with MPG at 75 mg/kg from day 4 were aseptically removed on days 1, 3, 5, 7, and 14. They were homogenized with a digital homogenizer (5,000 rpm, 30 *s*) in a guanidinium solution (5 M guanidinium thiocyanate, 31.25 mM sodium citrate, 0.625% Na-lauroylsarcosine, 0.12 mM β-mercaptoethanol, and antiform-A). The homogenates were kept at −80°C until use.

Histological examination. Hearts were fixed in 10% formalin, sectioned along the long axis through both the atria and ventricles, and stained with hematoxylin-eosin. Two sections per heart were examined by two observers who were unaware of any background data. The scores obtained for the extent of myocardial necrosis, inflammation, and calcification were averaged. Necrosis, inflammation, and calcification were scored from 0 to 4: grade 0 indicated no lesions or questionable lesions; grade 1, < 25% of the sampled myocardium contained lesions; grade 2, 3, and 4 indicated 25% increments.

RNA isolation. Total RNA was extracted from the above homogenates using the guanidinium/cesium chloride method (22) with some modifications. Briefly, the homogenates were centrifuged at 259,000 *g* for 10–12 h at 4°C through 5.7 M cesium chloride, subsequently deproteinization was done using phenol and chloroform/isoamyl alcohol plus precipitation with sodium acetate, SDS, and ethanol. The RNA yields were determined spectrophotometrically.

Northern blot analysis. Northern blot analysis was performed by a standard method (23) with some modifications. 10 μg of total RNA was denatured in 18 μl of formamide, formaldehyde, and Mops buffer (10× Mops; 200 mM Mops, 50 mM sodium acetate, and 10 mM EDTA, pH 7.0) at 65°C for 10 min. The RNA was fractionated by size on 1% agarose, 5.7% formaldehyde, 1× Mops buffer gel at 60 V, and transferred to a charged nylon membrane (Genescreenplus™; Dupont-New England Nuclear, Boston, MA) by passive capillary action in 10× SSC (1.5 M sodium chloride and 15 mM sodium citrate, pH 7.0). The membranes were baked at 80°C for 2 h, then prehybridized in 5× SSC, 5× Denhardt's solution (1% Ficoll 400, polyvinylpyrolidone, and BSA), 50% formamide, 1% SDS, 10% dextran sulphate, 50 mM sodium phosphate buffer (pH 6.8), and 250 μg/ml denatured salmon sperm DNA for 6–8 h at 42°C. The membranes were hybridized for 12–18 h at 42°C in the above hybridization solution with ^{32}P-labeled mouse Mn-SOD, mouse Cu/Zn-SOD, or mouse α-tubulin cDNAs. The ^{32}P-labeled cDNAs were products of random primer extension with Klenow fragment (Wako Pure Chemical Industries, Osaka, Japan) and [^{32}P]dCTP (3,000 Ci/mmol; Amersham International, Amersham, United Kingdom) to a specific activity of $> 10^8$ cpm/pmol DNA. Blots were washed twice in a covered container with 2× SSC and 0.1% SDS and twice with 0.2× SSC and 0.1% SDS at 60°C. They were then exposed for various periods to x-ray film at −70°C with intensifying screens. Densitometry was performed by Digital Densitorol (DMU-33C; Toyo Scientific Industries Co., Ltd., Tokyo, Japan).

Synthesis of cDNA probes and enzymatic amplification. Oligonucleotide primers for mouse Mn-SOD, Cu/Zn-SOD, and α-tubulin were designed and synthesized (Table I) (24–26). cDNAs were amplified by PCR using these oligonucleotide primers. The Mn-SOD, Cu/Zn-SOD, and α-tubulin cDNAs used as probes were 477, 441, and 288 bp, respectively. Protocol for PCR amplification was described elsewhere (27) with some modifications. Briefly, cDNA was synthesized in a 20-μl reaction volume containing 3 μg of total RNA from a noninfected mouse heart, 50 mM Tris-HCl, 60 mM KCl, 3 mM $MgCl_2$, 0.001% gelatin, 0.5 mM each dATP, dGTP, dTTP, and dCTP (Perkin Elmer Cetus Instruments, Norwalk, CT), 50 μg/ml oligo d(T) (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), and 20 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). The reaction was performed at 42°C for 1 h.

PCR amplification was done using the above reverse transcriptase mixture with 10× reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), a diluted mixture of deoxynucleotides, *Thermus aquaticus* DNA polymerase (GeneAmp Kit; Perkin Elmer Cetus Corp.), and each oligonucleotide primer. Amplification was programmed to include heat denaturation for 3 min at 94°C and then 40 PCR cycles (each cycle was 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min).

The PCR reaction products (60 μl) were electrophoresed at 100 V on 1.0% agarose gel in 0.2× TAE (1× TAE: 40 mM Tris acetate [pH 7.8], 1 mM EDTA). Gel was stained in ethidium bromide and DNA was detected by ultraviolet illumination at 302 nm. The band containing target cDNA was removed, cut into pieces, and then put into Su-

Table I. Oligonucleotide Sequences of 5′ and 3′ Primers for Polymerase Chain Reaction Amplification

mRNA	5′ Primer	3′ Primer	Product
			bp
Mn-SOD	$5'^{153}$GCTGGAGCCACACATTAACG	3′CGTGCGAATGATGGAAGTCA^{629}	477
Cu/Zn-SOD	$5'^{56}$ACCATCCACTTCGAGCAGAAGG	3′GGGACACACCAGACTTCAGAGT^{496}	441
α-Tubulin	$5'^{535}$AAGAAGTCCAAGCTGGAGTTC	3′GACTGTCTTAAGGTCTGGTTG^{822}	288

Superscripted number indicates the location of each cDNA.

prec®-1 tubes with filters (Takara Shuzo Co., Ltd., Kyoto, Japan). After being frozen at −20°C for 1 h and incubated at 37°C for 5 min, the gels were centrifuged at 5,000 *g* for 10 min at 4°C. The solution thus obtained was precipitated with sodium acetate, SDS, and ethanol. Recovered cDNA in pellets were dissolved in water and stored at −20°C until use.

Statistical analysis. The body weight, heart weight, and the ratios of heart weight to body weight were examined by one-way ANOVA for comparisons between each group and each experiment. Statistical analysis of the histological gradings of inflammation, necrosis, and calcification was performed by the same method. Survival of mice was analyzed by the Kaplan-Meier method.

Relative units of Mn- and Cu/Zn-SOD mRNA were also compared by one-way ANOVA. The unpaired and paired Student's *t* tests were used for some indexes, if necessary. Data are expressed as means±SD, except for the data in Figs. 3 *B* and 4 *B*.

Results

In our experimental murine model (28–30), viral replication in the heart is maximal on days 4 or 5 after inoculation. Myocardial virus titers then decrease and become very low by day 10. No virus can be isolated after day 14. There are few inflammatory cells, necrotic fibers, and dystrophic calcification in the heart on days 4 or 5, and the pathological changes subsequently become more extensive. On day 12, prominent myocardial calcification is noted and cellular infiltration is evident, consisting mainly of mononuclear cells. Mice that develop severe pathological damage, show congestive heart failure (severe congestion of the lungs and liver). Therefore, this model has two mortality peaks. The first, caused by viremia, is from days 4 to 5, and the second, caused by congestive heart failure, is from days 11 to 14.

Survival. Captopril experiment: On day 14, while 17 of 37 mice (46%) survived in the placebo group, 12 of 24 mice (50%) in the captopril 10 mg/kg group, 13 of 21 mice (62%) in the captopril 30 mg/kg group and 19 of 26 mice (73%) in the captopril 100 mg/kg group survived (Fig. 1). Thus, captopril improved survival of mice in a dose-dependent manner. Survival in the captopril 100 mg/kg group was significantly better than that in the placebo group from days 12 to 14 ($P < 0.05$).

MPG experiment: On day 14, while 17 of 30 mice (57%) survived in the placebo group, 24 of 30 mice (80%) in the MPG 8 mg/kg group, 21 of 25 mice (84%) in the MPG 25 mg/kg group and 22 of 25 mice (88%) in the MPG 75 mg/kg group survived. Survival in the MPG 8-, 25-, and 75-mg/kg groups was significantly better than that in the placebo group after days 10, 9, and 8, respectively ($P < 0.05$).

Histological examination. Cellular infiltration, myocardial necrosis, and calcification were seen on day 14 (Fig. 2). The histological scores of these elements in the captopril 30 mg/kg and 100 mg/kg groups were significantly lower than those in the placebo group. As seen in the captopril experiment, the scores of necrosis and infiltration in MPG 8 mg/kg group, and scores of three elements in MPG 25 mg/kg and MPG 75 mg/kg groups were significantly lower than those in the placebo group. In this way, the effects of MPG on survival and myocardial injury in virus-induced murine myocarditis were similar to those of captopril. The protective effect of captopril against the virus seemed to depend not on ACE inhibition but on its possession of a sulfhydryl group.

Body weight, heart weight, and the ratios of heart weight to body weight. In our model, infected mice lose body weight because of viremia in the acute stage and growth is inhibited by congestive heart failure in the subacute stage. So body weight of the placebo group was significantly lower than that of the uninfected groups in both the captopril and MPG experiments ($P < 0.01$). Treatment with captopril and MPG inhibited body weight loss of the mice (Table II). Body weight in the captopril 30 and 100 mg/kg groups and the MPG 25 or 75 mg/kg groups was significantly higher than that in the respective placebo groups ($P < 0.05$).

There was barely a significant difference in heart weight between the captopril 30 mg/kg group and the placebo group. On the other hand, the ratios of heart weight to body weight decreased dose dependently after each administration of captopril or MPG to a value similar to the uninfected normal group. There were significant differences in the ratios of heart weight to body weight between the placebo groups and the captopril 30 or 100 mg/kg groups, as well as the MPG 25 or 75 mg/kg groups ($P < 0.05$). Interestingly, the ratios of heart weight to body weight in the uninfected captopril treated group were significantly lower than in the uninfected normal group ($P < 0.05$, unpaired *t* test). Similar findings have also been observed in experiments using other ACE inhibitors (enalapril and cilazapril, unpublished data). However, there was no significant difference in the ratios of heart weight to body weight

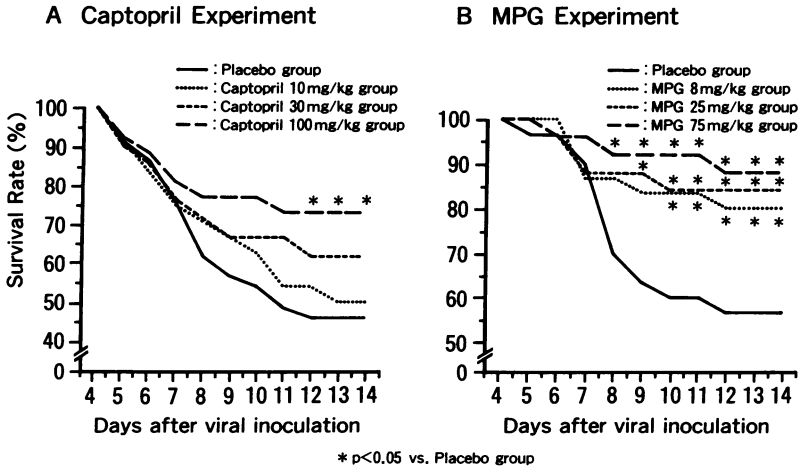

Figure 1. Effects of captopril and MPG on survival in murine myocarditis induced by encephalomyocarditis virus. The differences in survival became obvious after day 7. Survival in the captopril 100 mg/kg and MPG 75 mg/kg groups was significantly higher than that in the respective placebo groups after days 12 and 8.

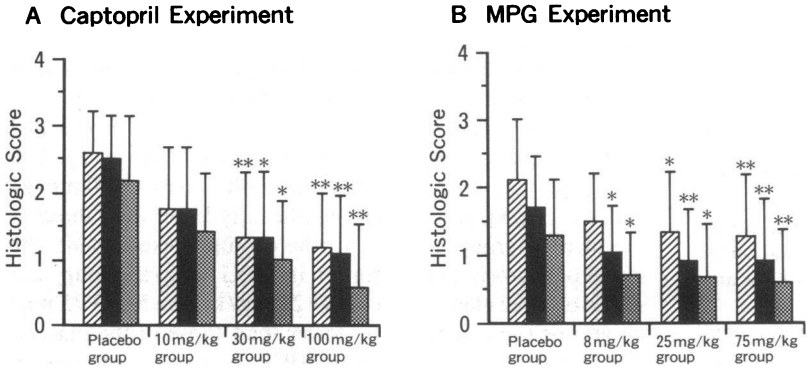

Figure 2. Effects of captopril and MPG on myocardial histopathology. The effects of captopril on the histological scores for cellular infiltration, necrosis, and calcification were similar to those of MPG. The number of mice examined is shown in Table II. $*P < 0.05$; $**P < 0.01$ vs. placebo group. ▨, Infiltration; ■, necrosis; ■, calcification.

between the uninfected normal group and the uninfected MPG treated group. The fact that MPG has no ACE inhibiting effect was supported by these findings. In spite of having no ACE inhibiting effect, the treatment with MPG reduced the ratios of heart weight to body weight of infected mice by its beneficial effects on myocardial damage.

Northern blot analysis. α-Tubulin was used as an internal control and relative units were calculated as ratios of the signal density for each SOD mRNA and α-tubulin mRNA. As shown in Figs. 3 and 4, mRNA for Mn- and Cu/Zn-SOD increased gradually in infected hearts, reached to maximum on day 5 after the viral inoculation, and then decreased to a level similar to that in uninfected hearts on day 14. Relative units of Mn-SOD mRNA expression in infected hearts were 1.0±0.2 on day 1, 5.6±1.6 on day 3, 9.9±1.8 on day 5, 4.4±0.9 on day 7, and 0.9±0.2 on day 14. In infected mice treated with MPG, cardiac expression of Mn-SOD mRNA decreased to a level similar to the uninfected mice (0.7±0.1 on day 5, 0.6±0.1 on day 7, and 0.7±0.2 on day 14). Relative units of Cu/Zn-SOD mRNA expression in infected hearts were 1.0±0.4 on day 1, 3.1±1.1 on day 3, 8.2±2.6 on day 5, 3.8±1.4 on day 7, and 1.5±0.4 on day 14. As with Mn-SOD, the expression of Cu/Zn-SOD mRNA decreased by MPG to a level similar to the uninfected hearts (0.8±0.2 on day 5, 0.7±0.1 on day 7, and 0.5±0.2 on day 14). Thus, dramatic induction of cardiac Mn- and Cu/Zn-SOD mRNA expression was demonstrated in response to viral infection. Furthermore, this induction of Mn- and Cu/Zn-SOD mRNAs was completely inhibited by MPG administration from day 4.

There were also changes in the expression of α-tubulin transcripts caused by viral infection (Table III *C*). α-Tubulin expression in infected hearts on days 3, 5, and 7 was decreased by about half compared with uninfected hearts. So α-tubulin seems to be inadequate for internal control. There were large variations in mRNA expression between mice, even when the time after inoculation was the same. We used α-tubulin in an attempt to correct these variations, indeed, the variations of the data corrected by α-tubulin could become small. The data uncorrected by α-tubulin are shown in Table III, *A* and *B*. The data uncorrected by α-tubulin showed results similar to those corrected by α-tubulin. Mn-SOD mRNA levels in infected hearts increased from day 3, became four- to sixfold higher than those in the uninfected hearts on day 5, and then decreased to the levels similar to the uninfected hearts on day 14. Cu/Zn-SOD mRNA levels also showed a two to fivefold increase compared with the uninfected hearts on day 5. However, expression of Cu/Zn-SOD mRNA in infected and uninfected hearts on day 3 was almost the same.

Table II. The Effects of Captopril and MPG on Body Weight, Heart Weight, and the Ratios of Heart Weight to Body Weight

Group	*n*	Body wt	Heart wt	Ratios of heart weight to body weight
		g	$\times 10^{-2}$ *g*	$\times 10^{-3}$
A. Captopril experiment				
Placebo group	12	12.1±1.8	6.76±1.14	5.65±0.84
10 mg/kg group	12	13.8±4.1	6.93±1.14	5.20±0.80
30 mg/kg group	13	15.8±3.1*	7.62±1.33*	4.88±0.63*
100 mg/kg group	12	15.4±3.9*	7.21±1.46	4.78±0.52*
Uninfected normal group	10	18.2±2.7**	8.26±1.04*	4.57±0.24**
Uninfected captopril-treated group	10	18.0±1.6**	7.35±0.98	4.07±0.33**
B. MPG experiment				
Placebo group	17	13.2±2.4	6.75±1.25	5.14±0.50
8 mg/kg group	24	13.5±3.2	6.36±1.35	4.77±0.67
25 mg/kg group	21	15.6±4.1*	7.42±1.85	4.76±0.35*
75 mg/kg group	22	15.4±3.5*	7.11±1.50	4.66±0.50*
Uninfected normal group	10	20.7±1.0**	9.53±0.56**	4.61±0.23*
Uninfected MPG-treated group	10	21.7±0.8**	9.90±0.68**	4.56±0.27*

* $P < 0.05$; ** $P < 0.01$ vs. placebo group.

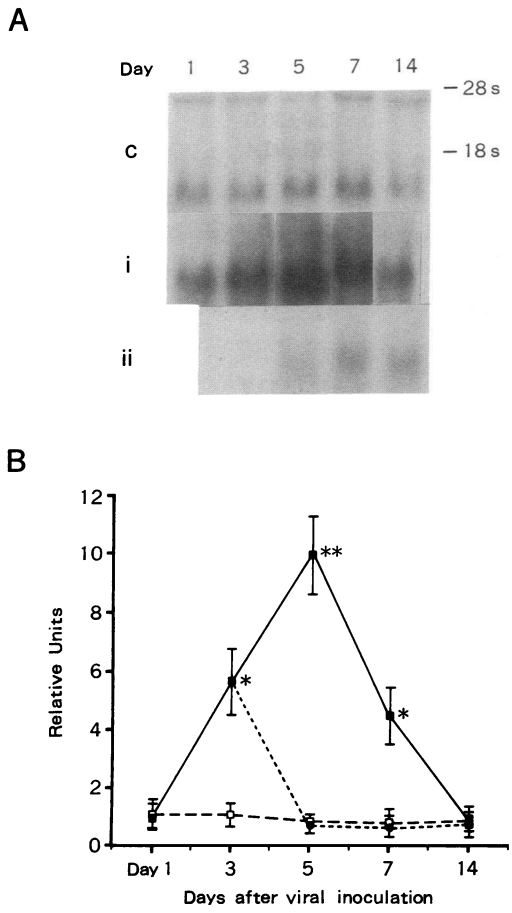

Figure 3. Northern blot analysis of Mn-SOD mRNA. (*A*) 10 μg of total RNA was isolated, fractionated by size, and transferred to a nylon membrane. The membrane was hybridized with ^{32}P-labeled Mn-SOD cDNA. Representative bands are shown from uninfected hearts (*C*), from infected hearts obtained on days 1, 3, 5, 7, and 14 (line *i*), and from infected hearts of mice treated with MPG (75 mg/kg) obtained on days 5, 7, and 14 (line *ii*). Our analysis of Mn-SOD had resulted in the identification of two classes of transcripts and we examined the smaller transcript. (*B*) Quantitative densitometry of Mn-SOD mRNA expression in each group. Relative unit indicates the ratio of Mn-SOD mRNA to α-tubulin mRNA. Data are expressed as means±SE. $n = 5$ in each group. $^*P < 0.05$; $^{**}P < 0.005$ vs. age-matched uninfected hearts. - - □ - -, Uninfected group; — ■ —, infected group; - - - ● - - -, treated group.

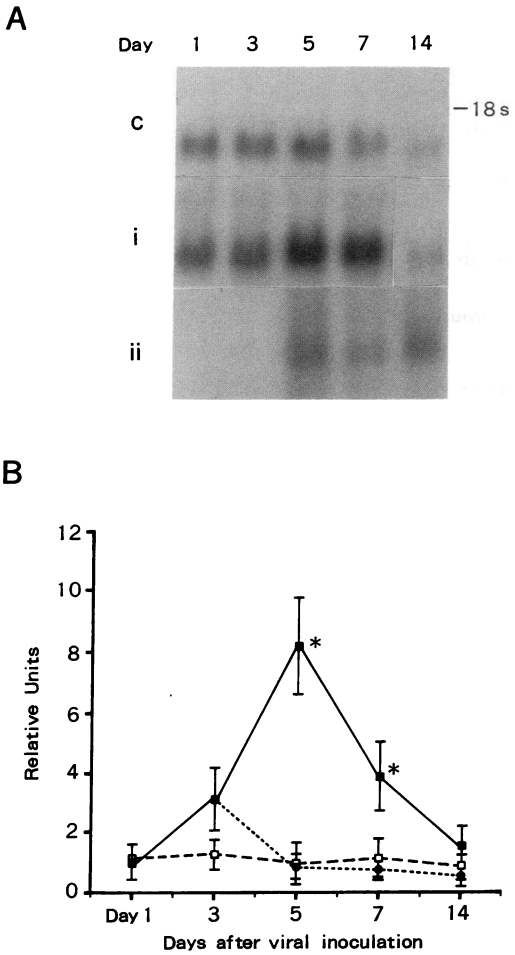

Figure 4. Northern blot analysis of Cu/Zn-SOD mRNA. (*A*) Representative bands are shown from uninfected hearts (*C*), from infected hearts obtained on days 1, 3, 5, 7, and 14 (line *i*), and from infected hearts of mice treated with MPG (75 mg/kg) obtained on days 5, 7, and 14 (line *ii*). (*B*) Quantitative densitometry of Cu/Zn-SOD mRNA expression in each group. Relative unit indicates the ratio of Cu/Zn-SOD mRNA to α-tubulin mRNA. Data are expressed as means ± SE. $n = 5$ in each group. $^*P < 0.05$ vs. age-matched uninfected hearts. - - □ - -, Uninfected group; — ■ —, infected group; - - ● - -, treated group.

Discussion

The oxygen free radical system is activated by influenza virus infection in the lungs (11). However, there is no report on the involvement of oxygen radicals in viral myocarditis. This study is the first to demonstrate the pathogenesis of oxygen radicals in virus-induced murine myocarditis by results, such as a striking increase of Mn- and Cu/Zn-SOD mRNA in infected hearts and the beneficial effects of sulfhydryl compounds against myocardial injury.

Both captopril and MPG improved survival, myocardial injury, and congestive heart failure in mice inoculated with encephalomyocarditis virus. On the other hand, enalapril, an ACE inhibitor without a sulfhydryl group, did not improve survival and myocardial injury although it improved congestive heart failure (10). Therefore, the beneficial effects of captopril were considered to be dependent not on its improvement of hemodynamics through ACE inhibition but on its possession of a sulfhydryl group.

Sulfhydryl compounds are well known for their antiinflammatory properties and have been reported to protect against irradiation- and drug-induced diseases in which free radicals have been implicated as mediators of tissue injury (31–33). Sulfhydryl compounds are thought to work by directly reacting with oxygen radicals, by promoting the resynthesis of glutathione, by providing an alternative substrate for glutathione peroxidase, or by reforming the disulfide cross-linking between proteins. Especially in myocardial ischemia/reperfusion injury mediated by oxygen radicals, sulfhydryl compounds including captopril and MPG have the protective effects (16–21, 34, 35). Captopril and MPG are possibly important in our model as free radical scavengers.

When oxygen radicals are produced beyond the capacity of

Table III. Quantitative Densitometric Analysis

Day	1	3	5	7	14
A. Mn-SOD					
Uninfected group	88.0±17.2	99.9±11.6	103±15.5	82.0±20.0	110±21.8
Infected group	105±26.6	238±11.3	504±45.4	291±92.4	113±16.3
MPG-treated group			60.9±10.0	39.5±15.1	36.4±12.5
B. Cu/Zn-SOD					
Uninfected group	97.0±15.3	123±36.8	112±41.8	117±16.2	107±33.6
Infected group	121±44.3	126±23.4	445±197	268±157	197±75.1
MPG-treated group			72.2±14.4	42.5±11.5	29.0±13.8
C. α-Tubulin					
Uninfected group	84.9±17.2	95.9±11.4	122±14.9	117±42.9	132±31.0
Infected group	100±10.0	48.0±20.7	52.5±10.5	66.5±19.2	127±24.9
MPG-treated group			88.3±16.4	60.6±16.2	51.8±10.6

The data were calculated as the densities of bands with the density of RNA expression from uninfected heart on day 3, which was common in every filter, being taken as 100. Data are expressed as means±SD. $n = 5$ in each group.

cells to eliminate them, they may cause serious damage. To protect cells from this damage, a family of SOD, catalase, and peroxidase serves as an intracellular defense mechanism against the overproduction of oxygen radicals. The first enzyme involved in the antioxidant defense system is SOD (30), which is specific for superoxide radicals as its catalytic substrate. High levels of Mn- and Cu/Zn-SOD mean an increased demand to eliminate superoxide radicals.

In this study, a striking increase of mitochondrial Mn-SOD and cytosolic Cu/Zn-SOD mRNA levels was seen in the virus-infected hearts, prompting us to hypothesize that overproduction of superoxide radicals was induced and that superoxide radicals play an important role in this disease. It was demonstrated that a sulfhydryl group did not directly scavenge superoxide radicals, but inhibited the superoxide radical generation in vitro (36). It is likely that MPG inhibits the increase in SOD mRNAs by eliminating superoxide radicals. Superoxide radicals were not examined directly, but our hypothesis may be supported by the fact that captopril and MPG with sulfhydryl groups improved myocardial injury, and that enalapril without a sulfhydryl group had no effect.

Induction of pathological damage by oxygen radicals. In influenza virus-infected lungs of mice, the enhancement of adenosine catabolism and oxygen radical generation was noted from day 4 after the virus inoculation and these changes paralleled the development of histological injury (11). Superoxide radicals, which were generated in the xanthine-xanthine oxidase system, directly induced pathological damage. However, the same system would not be activated in virus-induced myocarditis because the myocyte has no xanthine oxidase activity (37). Also, allopurinol, an inhibitor of xanthine oxidase, had no effect on myocardial injury in our murine model (unpublished data).

In our study, SOD mRNAs were maximal on day 5, and then the pathological changes increased, so there was a time lag between the changes in mRNA and pathological damage. If an increase of SOD mRNA reflects an increase of superoxide radicals, these radicals may not directly induce the pathological damage in the heart. Schreck et al. demonstrated that H_2O_2 induced the expression and replication of HIV and that hydroxy radicals served as intracellular messengers mediating the release of the inhibitory subunit IκB from NF-κB (38). In this study, superoxide radicals may produce H_2O_2 or hydroxy radicals as second messengers in the presence of increased SOD activity combined with decreased catalase and glutathione peroxidase activities. Then the hydroxy radicals may directly induce myocardial injury, or they may induce viral replication, resulting in cell damage or death. The changes of catalase and glutathione peroxidase activities still need to be clarified in this model. However, our hypothesis is supported by the fact that sulfhydryl compounds completely inhibited the increase of SOD mRNA and reduced myocardial injury in our model, presumably through the direct elimination of superoxide radicals and hydroxy radicals.

Production of oxygen radicals. When compared with the data uncorrected by α-tubulin (Table III, *A* and *B*), Mn- and Cu/Zn-SOD levels in infected hearts were maximal on day 5 and Mn-SOD levels in infected hearts were more than twice those in age-matched uninfected hearts on day 3. In contrast, Cu/Zn-SOD levels in the infected and uninfected hearts were almost the same on day 3. As Mn-SOD mRNA increased earlier than Cu/Zn-SOD mRNA, it seems likely that superoxide radicals were produced mainly in the mitochondria. An in vitro experiment showed that a selective Mn-SOD induction of mRNA in response to LPS and tumor necrosis factor had no effect on Cu/Zn-SOD in rat pulmonary epithelial cells (39). This suggests that the mitochondria have an important role in protection against oxygen radicals.

After picornavirus infection of mammalian cells, host cell RNA synthesis is rapidly inhibited to < 10% of the initial level (40). In cells infected with encephalomyocarditis virus, the activity of RNA polymerase I responsible for ribosomal RNA synthesis and that of RNA polymerase II transcribing mRNA were both inhibited, whereas RNA degradation was normal (41). α-Tubulin mRNA was likely to have been decreased by these mechanisms in our study. α-Tubulin may have been inadequate for the internal control; however, it is not easy to find a perfect one for our experimental conditions. The data uncorrected by α-tubulin showed results similar to those corrected by α-tubulin. We consider that our data showed a significantly increased expression of SOD mRNAs in the hearts infected with encephalomyocarditis virus.

We used MPG at doses of 8, 25, and 75 mg/kg to provide an amount of sulfhydryl groups equimolar to the doses of 10, 30, and 100 mg/kg of captopril. MPG has a longer blood half-life than captopril in humans (50 h after a single injection of 250 mg [42] and 2 h after a single injection of 10 mg [43]), and this may be why MPG was more effective in improving survival and preventing myocardial injury in our model.

In summary, our study showed a striking increase in Mn- and Cu/Zn-SOD mRNAs in virus-infected hearts. Both captopril and MPG were effective in inhibiting the changes due to viral myocarditis in our murine model. Oxygen radicals may play an important role in the pathogenesis of virus-induced myocarditis, and a therapeutic approach by eliminating oxygen radicals seems possible.

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References

1. Kawai, C., A. Matsumori, and H. Fujiwara. 1987. Myocarditis and dilated cardiomyopathy. *Annu. Rev. Med.* 38:221–239.

2. Abelmann, W. H., and B. H. Lorell. 1989. The challenge of cardiomyopathy. *J. Am. Coll. Cardiol.* 13:1219–1239.

3. Zee-cheng, C. S., C. C. Tsai, D. C. Palmer, J. E. Codd, D. G. Pennington, and G. A. Williams. 1984. High incidence of myocarditis by endomyocardial biopsy in patients with idiopathic congestive cardiomyopathy. *Am. J. Cardiol.* 3:63–70.

4. Matsumori, A., C. S. Crumpacker, and W. H. Abelmann. 1987. Prevention of viral myocarditis with recombinant human leukocyte interferon-α A/D in a murine model. *J. Am. Coll. Cardiol.* 9:1320–1325.

5. Sato, Y., S. Maruyama, C. Kawai, and A. Matsumori. 1992. Effect of immunostimulant therapy on acute viral myocarditis in an animal model. *Am. Heart J.* 124:428–434.

6. Matsumori, A., H. Wang, and W. H. Abelmann. 1985. Treatment of viral myocarditis with ribavirin in an animal preparation. *Circulation.* 7:834–839.

7. Suzuki, H., and A. Matsumori. 1990. Myocardial injury due to viral myocarditis improved by captopril in mice. *Circulation.* 82(Suppl III):III–674. (Abstr.)

8. Rezkalla, S., R. A. Kloner, G. Khatib, and R. Khatib. 1990. Beneficial effects of captopril in acute coxsackievirus B3 murine myocarditis. *Circulation.* 81:1039–1047.

9. Matsumori, A., H. Suzuki, and C. Kawai. 1990. Beneficial effects of captopril in coxsackievirus myocarditis. *Circulation.* 82:1882. (Letter to the editor.)

10. Suzuki, H., A. Matsumori, and C. Kawai. 1991. Effects of angiotensin converting-enzyme inhibitors on myocardial injury and congestive heart failure due to myocarditis in mice: comparison with captopril and enalapril. *Circulation.* 84(Suppl II):II–634. (Abstr.)

11. Akaike, T., M. Ando, T. Oda, T. Doi, S. Ijiri, S. Araki, and H. Maeda. 1990. Dependence on O_2^- generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. *J. Clin. Invest.* 85:739–745.

12. Freeman, B. A., and J. D. Crapo. 1982. Biology of disease. Free radicals and tissue injury. *Lab. Invest.* 47:412–426.

13. MaCord, J. M. 1985. Oxygen-derived free radicals in post-ischaemic tissue injury. *N. Engl. J. Med.* 312:159–163.

14. MaCord, J. M. 1988. Free radicals and myocardial ischaemia: overview and outlook. *Free Rad. Biol. Med.* 4:9–14.

15. Przyklenk, K., and R. A. Kloner. 1989. Relationships between structure and effects of ACE inhibitors: comparative effects in myocardial ischaemic/reperfusion injury. *Br. J. Pharmacol.* 28:167s–175s.

16. Gilst, W. H., P. A. Graeff, H. Wesseling, and C. D. J. deLangen. 1986. Reduction of reperfusion arrhythmias in the ischemic isolated rat heart by angiotensin converting enzyme inhibitors: a comparison of captopril, enalapril, and HOE 498. *J. Cardiovasc. Pharmacol.* 8:722–728.

17. Pi, X., and X. Chen. 1989. Captopril and ramiprilat protect against free radical injury in isolated working rat hearts. *J. Mol. Cell. Cardiol.* 21:1261–1271.

18. Mitsos, S. E., T. E. Askew, J. C. Fantone, S. L. Kunkel, G. D. Abrams, A. Schork, and B. R. Lucchesi. 1986. Protective effects of N-2-mercaptopropionyl glycine against myocardial reperfusion injury after neutrophil depletion in the dog: evidence for the role of intracellular-derived free radicals. *Circulation.* 73:1077–1086.

19. Mitsos, S. E., J. C. Fantone, K. P. Gallagher, K. M. Walden, P. J. Simpson, G. D. Abrams, M. A. Schork, and B. R. Lucchesi. 1986. Canine myocardial reperfusion injury: protection by a free radical scavenger, N-2-mercaptopropionyl glycine. *J. Cardiovasc. Pharmacol.* 8:978–988.

20. Zimmer, G., and J. Evers. 1988. 2-Mercaptopropionylglycine improves aortic flow after reoxygenation in working rat hearts. *Basic. Res. Cardiol.* 83:445–451.

21. Westlin, W., and K. Mullane. 1988. Does captopril attenuate reperfusion-induced myocardial dysfunction by scavenging free radicals? *Circulation.* 77(Suppl I.):I30–I39.

22. Chirgwin, J. J., A. E. Prezbyla, R. J. Macdonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

23. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1991. Current protocols in molecular biology. Green Publishing Associates and Wiley-Interscience, New York. 4.9.1–4.9.7.

24. Hallewell, R. A., G. T. Mullenbach, M. M. Stempien, and G. I. Bell. 1986. Sequence of a cDNA coding for mouse manganese superoxide dismutase. *Nucleic Acids Res.* 14:9539.

25. Bewley, G. C. 1988. cDNA and deduced amino acid sequence of murine Cu-Zn superoxide dismutase. *Nucleic Acids Res.* 16:2728.

26. Lewis, S. A., M. G. Lee, and N. J. Cowan. 1985. Five mouse tubulin isotypes and their regulated expression during development. *J. Cell Biol.* 101:852–861.

27. Kyu, B. S., A. Matsumori, Y. Sato, I. Okada, N. M. Chapman, and S. Tracy. 1992. Cardiac persistence of cardioviral RNA detected polymerase chain reaction in a murine model of dilated cardiomyopathy. *Circulation.* 86:522–530.

28. Tomioka, N., C. Kishimoto, A. Matsumori, and C. Kawai. 1986. Effects of prednisolone on acute myocarditis in mice. *J. Am. Coll. Cardiol.* 7:868–872.

29. Matsumori, A., and C. Kawai. 1982. An experimental model for congestive heart failure after encephalomyocarditis virus myocarditis in mice. *Circulation.* 65:1230–1235.

30. Matsumori, A., and C. Kawai. 1982. An animal model of congestive (dilated) cardiomyopathy: dilatation and hypertrophy of the heart in the chronic stage in DBA/2 mice with myocarditis caused by encephalomyocarditis virus. *Circulation.* 66:355–360.

31. Doroshow, J. H., G. Y. Locker, I. Ifrim, and C. E. Myers. 1981. Prevention of doxorubicin cardiac toxicity in the mouse by *N*-acetyl-cysteine. *J. Clin. Invest.* 68:1053–1064.

32. Devi, P. U., and P. G. S. Prasanna. 1990. Radioprotective effects of combinations of WR-2721 and mercaptopropionylglycine on mouse bone marrow chromosomes. *Radiat. Res.* 124:165–170.

33. Forman, H. J., E. I. Rotman, and A. B. Fisher. 1983. Roles of selenium and sulfur-containing amino acids in protection against oxygen toxicity. *Lab. Invest.* 49:148–153.

34. Ferrari, R., C. Ceconi, S. Curello, A. Cargnoni, A. Pardini, P. Marzollo, and O. Visioli. 1991. Oxygen free radicals and myocardial damage: protective role of thiol-containing agents. *Am. J. Med.* 91(Suppl. 3c):3c95s–3c105s.

35. Koerner, J. E., B. A. Anderson, and R. C. Dage. 1991. Protection against postischemic myocardial dysfunction in anesthetized rabbits with scavengers of oxygen-derived free radicals: superoxide dismutase plus catalase, N-2-mercaptopropionyl glycine and captopril. *J. Cardiovasc. Pharmacol.* 17:185–191.

36. Mehta, J. L., F. A. Nicolini, and D. L. Lawson. 1990. Sulfhydryl group angiotensin converting-enzyme inhibitors and superoxide radical formation. *J. Cardiovasc. Pharmacol.* 16:847–849.

37. Eddy, L. J., J. R. Stewart, H. P. Jones, T. D. Engerson, J. M. McCord, and J. M. Downey. 1987. Free radical producing enzyme, xanthine oxidase, is undetectable in human hearts. *Am. J. Physiol.* 253:H709–H711.

38. Shreck, R., P. Rieber, and P. A. Baeuerle. 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2247–2258.

39. Visner, G. A., W. C. Dougall, J. M. Wilson, I. A. Burr, and H. S. Nick. 1990. Regulation of manganese superoxide dismutase by lipopolysaccharide, interleukin-1, and tumor necrosis factor. *J. Biol. Chem.* 265:2856–2864.

40. Kaariainen, L. 1984. Inhibition of cell functions by RNA-virus infections. *Annu. Rev. Microbiol.* 38:91–109.

41. Schwartz, L. B., C. Lawrence, R. E. Thach, and R. G. Roeder. 1974. Encephalomyocarditis virus infection of mouse plasmacytoma cells. II. Effect on host RNA synthesis and RNA polymerases. *J. Virol.* 14:611–619.

42. Carlsson, S. M., T. Denneberg, B. M. Emanuelsson, B. Kågedal, and S. Lindgren. 1990. Pharmacokinetics of intravenous 2-mercaptopropionylglycine in man. *Eur. J. Clin. Pharmacol.* 38:499–503.

43. Duchin, K. L., S. M. Singhvi, D. A. Willard, B. H. Migdalof, and D. N. McKinstry. Captopril kinetics. *Clin. Pharmacol. & Ther.* 31:452–458.