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Alterations in cellular Ca^{2+} and free iron pool by sulfur amino acid deprivation: the role of ferritin light chain down-regulation in prooxidant production

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Abstract

Deficiency of sulfur amino acids occurs in certain pathophysiological states such as protein-calorie malnutrition. Sulfur amino acid deprivation (SAAD) increases oxidative stress through a decrease in GSH. Ferritin expression is induced by oxidative stress, which confers resistance to oxidative insults. The effects of SAAD on the changes in cellular Ca^{2+} and free iron pool, prooxidant production and the ferritin light chain (FLC) expression were comparatively evaluated in Hepa1c1c7 and Raw264.7 cells. $[Ca^{2+}]_i$ was rapidly increased by SAAD. Sulfhydryl-containing compounds prevented the increase in $[Ca^{2+}]_i$ in cells under SAAD, supporting the role of redox-state in the regulation of $[Ca^{2+}]_i$. Thapsigargin or Ca^{2+}-free medium inhibited the increase in $[Ca^{2+}]_i$, showing that Ca^{2+} originated from endoplasmic reticulum as well as from extracellular source. Inhibition of Ca^{2+} mobilization decreased the fluorescence of Phen Green SK inside cells, representing the inhibition of free iron release. Both inhibition of Ca^{2+} mobilization and iron chelation decreased dichlorofluorescein oxidation, indicating the possibility that the increase in $[Ca^{2+}]_i$ affected that in cellular free iron and prooxidant production. FLC protein level was immunochemically detectable in Raw264.7 cells, but not in Hepa1c1c7 cells. SAAD alone (or in combination with $FeSO_4$) down-regulated FLC protein expression, while SAAD increased the FLC mRNA level in both Hepa1c1c7 and Raw264.7 cells. Calcium or iron chelators prevented increases in the FLC mRNA. These results provided evidence that changes in cellular Ca^{2+} and iron pool by SAAD increased cellular oxidative stress and that the down-regulation of FLC protein by SAAD would further enhance prooxidant production in spite of the increase in FLC mRNA.

Keywords: Ferritin light chain; Calcium; Sulfur amino acids; Oxidative stress

1. Introduction

Deficiency of sulfur amino acids occurs in certain pathophysiological states such as protein-calorie malnutrition. Previous studies from this laboratory have shown that protein-calorie malnutrition induces oxidative stress through a persistent decrease in cellular GSH content as a result of sulfur amino acid deficiency [1,2]. Cells exposed to sulfur amino acid deprivation (SAAD) exhibit the pathophysiological properties similar to those of protein-calorie malnutrition [3]. Lack of sulfur amino acids in culture medium causes a decrease in cellular GSH, which would affect the cellular redox state and the activities of sulfhydryl-containing enzymes. A previous study has shown that exposure of cells to SAAD decreases cellular GSH and increases oxidative stress [3]. SAAD-induced oxidative stress differs from that induced by GSH depleting agents in the time course of GSH depletion. SAAD persistently disrupts a dynamic equilibrium of the GSH pool and limits the compensatory increase in GSH synthesis.

Oxidative stress induces biological responses by oxidant-mediated activation of Ca^{2+}-signaling and stimulation of signal transduction components [4]. Previously, we have shown that SAAD activates all three MAP kinases ERK1/2, p38 kinase and JNK [3,5]. The present study was designed to investigate the effects of SAAD on the cell type-specific alterations in $[Ca^{2+}]_i$ and free iron pool and prooxidant production. In the current study, we showed that an increase in $[Ca^{2+}]_i$ by SAAD preceded that in cellular free iron release, which was apparently responsible for prooxidant production.

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Abbreviations: DCF, dichlorofluorescein; DFO, deferoxamine; FLC, ferritin light chain; MEM, minimal essential medium; ROS, reactive oxygen species; SAAD, sulfur amino acid deprivation; SSC, standard saline citrate.

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Ferritin, a multimeric protein composed of heavy and light subunits, stores iron in a non-toxic form and limits the reactivity of cellular iron [6,7]. Although, ferritin as a natural iron chelator protects cells from injury, it is still controversial whether ferritin represents prooxidant or antioxidant. Although, early degradation of ferritin expands cellular free iron pool, activation of ferritin expression at later times may limit the prooxidant challenge [8]. Oxidative stress affects the expression of antioxidant genes including ferritin light chain (FLC). Chemicals generating oxidative stress increase the synthesis of ferritin and transcriptionally activate the gene as a compensatory response [8]. A change in free iron availability also regulates the ferritin gene expression [9]. A number of studies have shown that the expression of FLC is under the translation control in response to free iron [10–14]. Ferritin expression is translationally controlled through interaction of an iron regulatory factor with the iron-responsive element in the 5′-untranslational region of the mRNA [15,16]. Nonetheless, the FLC protein expression in response to oxidative stress, in particular to deficiency of sulfur amino acids, has not been studied yet. We were interested in establishing the effect of SAAD on the FLC expression in association with cellular Ca^{2+} and free iron changes in hepatocyte- and monocyte-derived murine cell lines. In the present study, we chose these two cell lines to comparatively evaluate the extents of cellular oxidative stress and FLC expression in response to SAAD, because the total cellular iron and iron storage capacities of hepatocytes and macrophages differ each other. We found that SAAD down-regulated FLC expression in spite of the increase in its mRNA. Diminished iron storage capacity due to suppression of FLC protein may be the oxidative risk factor for cells in deficiency of sulfur amino acids.

2. Materials and methods

2.1. Materials

[α-^{32}P]dCTP (3000 mCi/mmol) was purchased from Perkin-Elmer Life Sciences. Minimum essential medium (MEM)-select amine kit was obtained from Life Technologies. Calcium Green-1 AM and Phen Green SK dipotassium were purchased from Molecular Probes. A rabbit anti-human ferritin antibody and all other chemicals were purchased from Sigma.

2.2. Cell culture

Hepa1c1c7 and Raw264.7 cell lines were obtained from Korean Cell Line Bank and maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin at 37° in humidified atmosphere with 5% CO_2. Sulfur amino acid-deprived MEM was reconstituted with Earle's balanced salt solution, vitamin mixture and the amino acids except cystine and methionine [3]. Cells were incubated for the indicated times in the reconstituted MEM with or without cystine and methionine. For storage of cells, cells were frozen at −150°.

2.3. Prooxidant production

Production of intracellular peroxides was monitored spectrofluorometically using dichlorofluorescein diacetate (DCFH-DA) as a fluorescent dye [17]. Cells were suspended 12 hr after serum deprivation and then DCFH-DA dissolved in ethanol was added at the final concentration of 10 μM in the medium. Oxidation of DCFH by peroxides yielded dichlorofluorescein (DCF). Fluorescence was monitored at the excitation and emission wavelengths of 485 and 530 nm, respectively using a fluorescence plate reader (50 cycles per 20 s at 37°) (Tecan®, Tecan US Inc.). Data were expressed as relative changes to the initial fluorescence.

2.4. Assay of $[Ca^{2+}]_i$

Intracellular calcium content was assayed as previously described by Silei *et al.* [18]. Briefly, cells (1×10^5) were attached to Lab-TEK chamber slides® (Nalge Nunc International Corp.) for 6 hr in serum-free medium. Cells were exposed to Calcium Green-1 AM at a final concentration of 5 μM and incubated at 37° for 45 min. After dye loading, cells were washed three times with PBS to remove excess dye. Fluorescence was monitored at the excitation wavelength of 488 nm and the emission wavelength of 530 nm using a confocal microscope (Leica TCS NT, Leica Microsystems). The intracellular calcium level was shown as F/F_o. $F/F_o = (\text{absorbance} - \text{background absorbance})/(\text{zero-time absorbance} - \text{background absorbance})$ [19]. The peak value of F/F_o in the present study was highly reproducible when measured up to 200 s. Multiple analyses were carried out to determine relative changes.

2.5. Determination of chelatable cellular iron

Cells suspended in MEM were loaded with 20 μM Phen Green SK a fluorescent iron chelator for 15 min, as described previously [20,21]. After dye loading, cells were washed three times with PBS. Fluorescence of Phen Green SK, which represented the release of free iron, was monitored at the excitation wavelength of 488 nm and the emission wavelength of 530 nm using a confocal microscope.

2.6. Preparation of a cDNA probe for FLC

The specific cDNA probe for the mouse *FLC* gene was amplified by reverse transcription-polymerase chain reaction using the selective primers (forward primer,

5′-TACAAGTCTCTCCAGTCG-3′; reverse primer, 5′-CTAGTCGTGCTTGAGAGT-3′) [22] and was cloned in the Topo TA cloning vector (Invitrogen).

2.7. Northern blot hybridization

The total RNA was isolated from cells using the improved single-step method of thiocyanate–phenol–chloroform RNA extraction and Northern blot analysis was carried out according to the procedures described previously [23]. Briefly, the total RNA was resolved by electrophoresis in a 1% agarose gel containing 2.2 M of formaldehyde and transferred to nitrocellulose paper. The nitrocellulose paper was baked in a vacuum oven at 80° for 2 hr. The blot was incubated with hybridization buffer containing 50% deionized formamide, 5 × Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrrolidine and 0.1% bovine serum albumin (Pentex Fraction V)], 0.1% SDS, 200 μg/mL of sonicated salmon sperm DNA and 5 × SSPE (1 × SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4 and 1 mM Na_2EDTA, pH 7.4) at 42° for 1 hr without probe. Hybridization was performed at 42° for 18 hr with a heat-denatured cDNA probe, which was random prime-labeled with [α-^{32}P]dCTP. Filters were washed in 2 × SSC and 0.1% SDS for 10 min at room temperature twice and in 0.1 × SSC and 0.1% SDS for 10 min at room temperature twice. Filters were washed in the solution containing 0.1 × SSC and 0.1% SDS at 60° for 60 min. After quantification of mRNA levels, the membranes were stripped and rehybridized with a [^{32}P]-labeled cDNA probe complementary to 18S rRNA to quantify the amount of RNA loaded onto the membranes.

2.8. Western blot analysis of FLC

After washing the cells twice with PBS, the total cell lysates were prepared by lysing cells in the buffer containing 50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride, 2 μg/mL aprotinin, 10 μg/mL leupeptin and 1 mM sodium orthovanadate. The cell lysates were centrifuged at 12,000 *g* for 10 min to remove debris. Cell lysates were separated by 12% gel electrophoresis and electrophoretically transferred to nitrocellulose paper. Western blot analysis was performed, as described previously [24]. FLC was immunochemically assessed using rabbit anti-human ferritin antibody (1:16 dilution), which recognized ferritin light and heavy chains and developed using ECL chemiluminescence system (Amersham). The antibody was capable of immunoprecipitating ferritin light and heavy subunits specifically. The immunoprecipitate was used as a ferritin standard, which identically migrated with those detected by Western blot analysis.

2.9. Data analysis

Scanning densitometry was performed with Image Scan & Analysis System (Alpha-Innotech Corporation). One-way ANOVA procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman–Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Increase in $[Ca^{2+}]_i$ by SAAD

Calcium homeostasis is closely associated with oxidative stress. We were interested in whether the change in redox state by SAAD altered Ca^{2+} homeostasis. We first determined cellular Ca^{2+} in Hepa1c1c7 cells using Calcium Green-1 AM, a Ca^{2+}-binding dye. When Hepa1c1c7 cells were exposed to SAAD medium, $[Ca^{2+}]_i$ rapidly increased for the first 100 s. The Ca^{2+} level remained

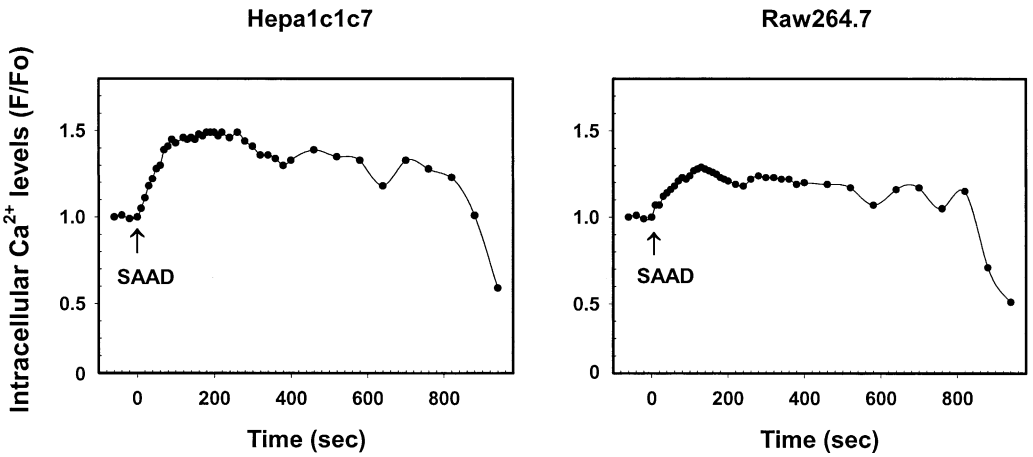

Fig. 1. Changes in $[Ca^{2+}]_i$ in Hepa1c1c7 and in Raw264.7 cells. When cells were exposed to SAAD medium, $[Ca^{2+}]_i$ rapidly increased in Hepa1c1c7 cells. The relative increase in $[Ca^{2+}]_i$ in Raw264.7 cells was less than that in Hepa1c1c7 cells. Data represent mean from three separate experiments.

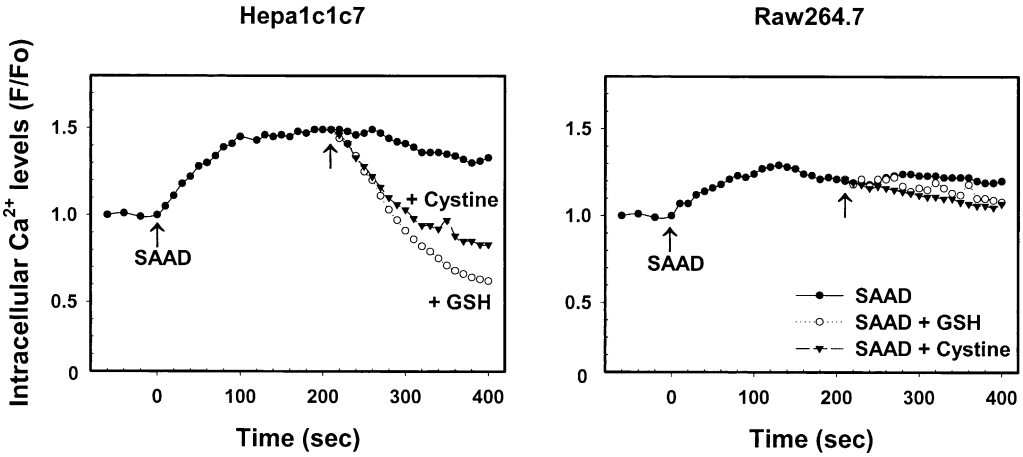

Fig. 2. The effects of GSH or cystine on the increase in $[Ca^{2+}]_i$ by SAAD. The cells were exposed to 5 mM of GSH or cystine following incubation in SAAD medium for the first 200 s. Data represent mean from three separate experiments.

elevated up to 800 s, a limit of assaying time under the confocal microscope (Fig. 1). In particular, $[Ca^{2+}]_i$ was 1.5-fold increased at 100–400 s. Intracellular Ca^{2+} was also increased by SAAD in Raw264.7 cells, the extent of which was less than that in Hepa1c1c7 cells (Fig. 1).

The effects of GSH and cystine on the increase in $[Ca^{2+}]_i$ were monitored in Hepa1c1c7 and Raw264.7 cells. When the cells in SAAD medium were exposed to 5 mM GSH or cystine, $[Ca^{2+}]_i$ rapidly declined (Fig. 2). Cysteine was also active (data not shown). $[Ca^{2+}]_i$ in control cells was also decreased by the presence of GSH presumably due to a change in the redox state (data not shown). Either GSH or cystine was marginally active in reversing $[Ca^{2+}]_i$ increase in Raw264.7 cells probably because the rise in $[Ca^{2+}]_i$ in macrophages was not as much distinct as that in Hepa1c1c7 cells. These results showed that SAAD caused an increase in cellular Ca^{2+} in both cell types via alteration in the redox state.

Whether the cellular Ca^{2+} originated from extracellular source or from intracellular storage was determined. Exposure of Hepa1c1c7 cells to Ca^{2+}-free SAAD medium resulted in a smaller increase in $[Ca^{2+}]_i$. Treatment of cells with 2 μM verapamil for 5 min also reduced the extent of $[Ca^{2+}]_i$ increase by SAAD. Thapsigargin (1 μM, 5 min-preincubation), which depleted intracellular storage of calcium through inhibition of Ca^{2+}-ATPase in endoplasmic reticulum, inhibited the rise of $[Ca^{2+}]_i$ by SAAD (Fig. 3). A preliminary study showed that a 5 min-incubation with thapsigargin was sufficient to deplete cellular stored $[Ca^{2+}]_i$. These results raised the possibility that SAAD increased $[Ca^{2+}]_i$ through stimulation of both endoplasmic reticulum and plasma membrane.

3.2. Role of Ca^{2+} in the increase of cellular free iron

We were interested in whether the increase in $[Ca^{2+}]_i$ by SAAD led to a change in free iron release in cells. Cellular free iron increased after exposure of Phen Green SK-loaded Hepa1c1c7 cells to SAAD medium. Fluorescence of Phen Green SK was detectable at 20–30 min, which

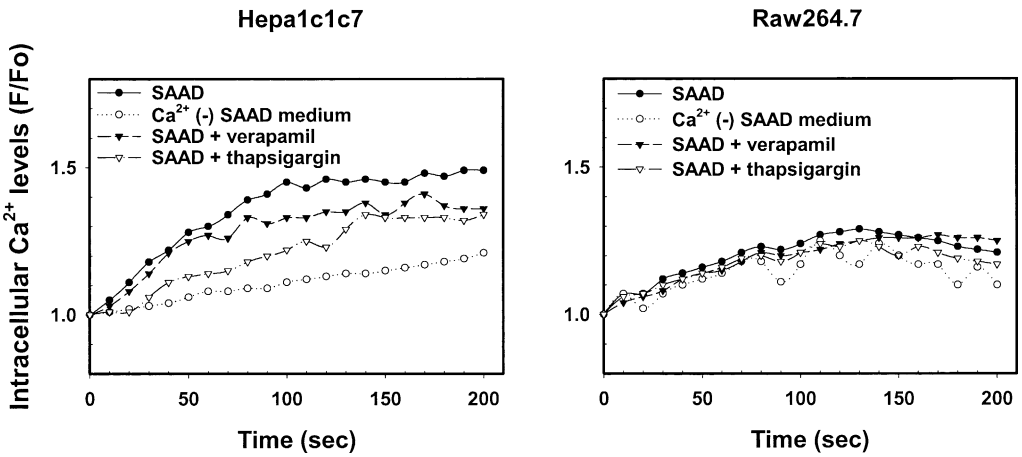

Fig. 3. The effects of Ca^{2+}-free SAAD medium or Ca^{2+} blockers on the increase in $[Ca^{2+}]_i$ by SAAD. $[Ca^{2+}]_i$ was monitored using the fluorescence of 5 μM Calcium Green-1 AM in Hepa1c1c7 or Raw264.7 cells incubated in control and SAAD medium. The cells were incubated in the presence of 2 μM verapamil for 5 min or 1 μM thapsigargin for 5 min. Subsequently, cells were exposed to SAAD medium to determine $[Ca^{2+}]_i$. Data represent the mean from three separate experiments.

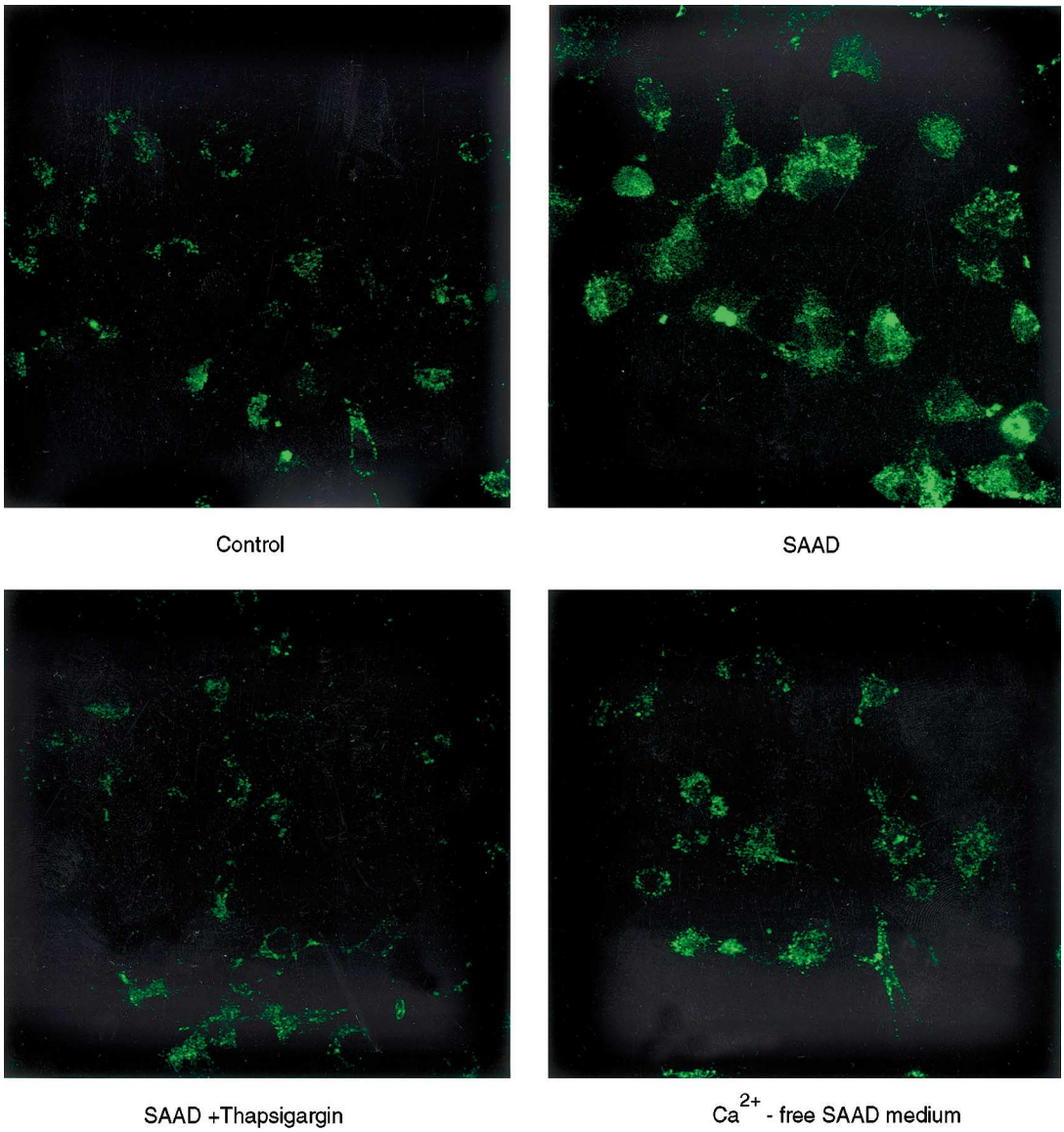

Fig. 4. Representative photographs showing the release of cellular free iron in dye-loaded Hepa1c1c7 cells. The cells were loaded with 20 μM Phen Green SK, a fluorescent iron chelator, for 15 min. After dye loading, cells were exposed to SAAD medium for 1 hr following incubation with or without 1 μM thapsigargin for 5 min or to Ca^{2+}-free SAAD medium for 1 hr. Fluorescence was monitored at the excitation wavelength of 488 nm and the emission wavelength of 530 nm using a confocal microscope. Results were confirmed by three repeated experiments.

represented release of free iron in cells. The increase in free iron extended up to 100 min (the limit of assaying time), as evidenced by fluorescence microscopy. Cellular iron notably increased in Hepa1c1c7 cells under SAAD medium for 1 hr, as compared with control (Fig. 4). Although, the release of free iron was increased by SAAD in Raw264.7 cells, the fluorescence change was very weak, as compared to that in Hepa1c1c7 cells (data not shown). We determined whether the rise in $[Ca^{2+}]_i$ by SAAD, which was observed at least for the first 10 min, stimulated the release of free iron in dye-loaded Hepa1c1c7 cells. Cells exposed to SAAD for 1 hr following incubation with thapsigargin for 5 min showed no increase in cellular free iron (Fig. 4). Role of Ca^{2+} in the increase in free iron was further confirmed by no increase in iron release in cells incubated in Ca^{2+}-free SAAD medium (Fig. 4). These results demonstrated that the increase in $[Ca^{2+}]_i$ by SAAD was responsible for the release of free iron.

3.3. Role of Ca^{2+} in prooxidant production

We then determined fluorescence of oxidized DCF in dye-loaded Hepa1c1c7 and Raw264.7 cells cultured in SAAD medium with or without extracellular Ca^{2+} or 2 μM verapamil. SAAD markedly increased the rate of DCF oxidation in Hepa1c1c7 cells (Fig. 5). The extent of DCF oxidation for the first 5 min was greater in Hepa1c1c7 cells than in Raw264.7 cells (Fig. 5). Verapamil inhibited prooxidant production in Hepa1c1c7 cells to a certain extent, which was comparable to that in cells under

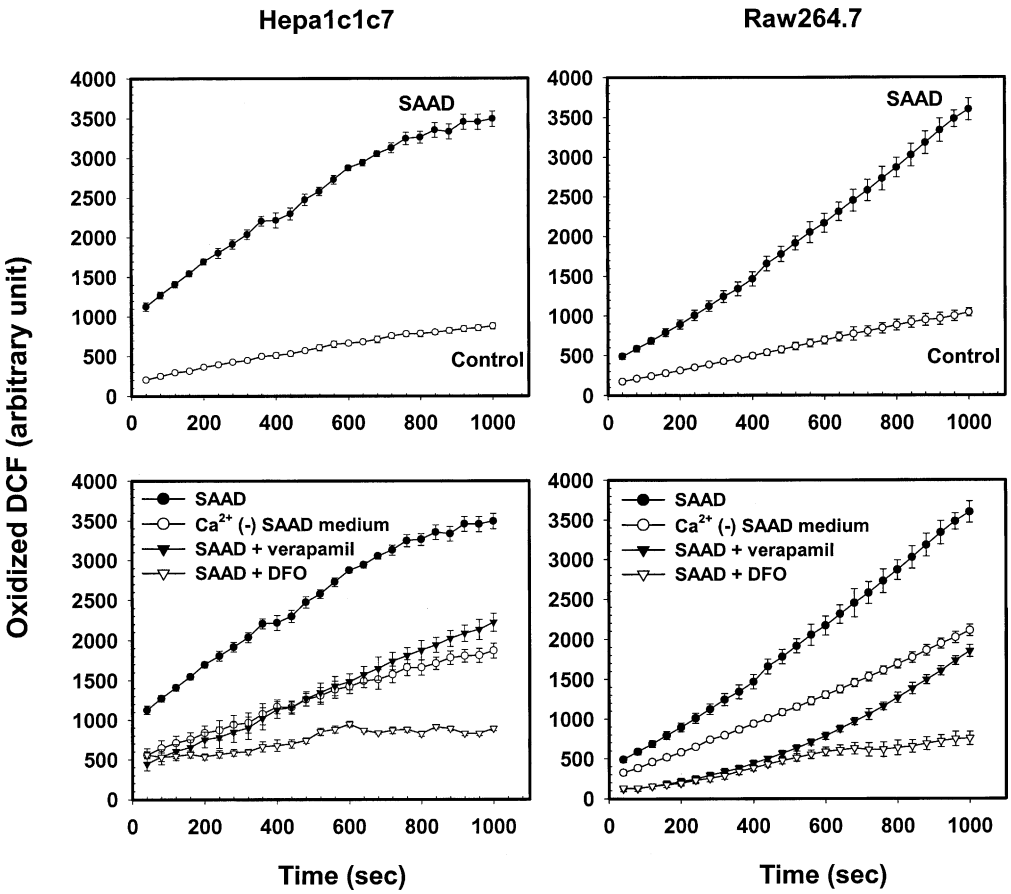

Fig. 5. Fluorescence of oxidized DCF in Hepa1c1c7 or in Raw264.7 cells cultured in control or SAAD medium. The cells were preincubated without serum for 12 hr and then loaded with 10 μM DCFH-DA. Dye-loaded cells were subjected to control or SAAD medium with or without extracellular Ca^{2+} or 2 μM varapamil. Fluorescence of DCF was monitored at the excitation wavelength of 485 nm and the emission wavelength of 530 nm. Data represent the mean ± SE with four separate experiments.

Ca^{2+}-free SAAD medium. The rate of DCF production was also increased by SAAD in Raw264.7 cells, which was inhibited by Ca^{2+}-free condition or by the presence of verapamil (at early times) (Fig. 5). The increase in slope in the presence of verapamil after 600 s may have resulted from a compensatory increase in cellular calcium. The extent of DCF production did not exactly match with the change in $[Ca^{2+}]_i$ in Raw264.7 cells (Fig. 3 vs. Fig. 5). The difference may result from the cell type-specific differences in dye loading and/or gradual changes in the expression of antioxidant enzymes (e.g. FLC protein).

3.4. Effect of free iron on prooxidant production

Studies have shown that oxidative stress (e.g. reactive oxygen species) was highly associated with an increase in cellular free iron pool [7,25]. To assess whether an increase in free iron was directly responsible for prooxidant production, the cells were incubated in control or SAAD medium in the presence of deferoxamine (DFO), an iron chelator (Table 1). DFO was active in inhibiting prooxidant production in a concentration-dependent manner in Hepa1c1c7 cells in control or SAAD medium for 5–10 min. DCF oxidation was also inhibited in Raw264.7 cells to similar extents (Table 1). Unlike the partial suppression of oxidative stress by Ca^{2+}-free condition (Fig. 4), DFO chelation of iron was more effective in suppressing prooxidant production. Hence, the rise of $[Ca^{2+}]_i$ might serve only as an initiating signal for oxidative stress. Rather, cellular free iron appeared to be ultimately responsible for SAAD-induced prooxidant production in both cell types.

3.5. Expression of FLC protein

Ferritin consisting of light and heavy chains is an iron-binding protein inside the cells. The expression of FLC was quantified in Hepa1c1c7 and Raw264.7 cells under SAAD. The anti-ferritin antibody immunoprecipiated ferritin light and heavy chains only in lysates obtained from Raw264.7 cells. Western blot analysis was employed for the subsequent experiments. Both ferritin light and heavy chains in Raw264.7 cells were detectable by Western blot analysis (Fig. 6). Either Western blotting or immunoprecipitation analysis failed to detect FLC protein in Hepa1c1c7 cells (data not shown). Treatment of Raw264.7 cells with 100 μM $FeSO_4$ for 12 hr caused an increase in FLC protein level (i.e. 140% of control). The level of FLC protein,

Table 1
The effects of DFO on prooxidant production

Treatment	Hepa1c1c7 cells				Raw264.7			
	5 min		10 min		5 min		10 min	
	DCF oxidation (arbitrary unit)	Change (%)	DCF oxidation (arbitrary unit)	Change (%)	DCF oxidation (arbitrary unit)	Change (%)	DCF oxidation (arbitrary unit)	Change (%)
Control medium	438 ± 17	100	664 ± 25	100	406 ± 27	100	692 ± 52	100
Control medium + DFO 2 μM	346 ± 18**	79	525 ± 28**	79	382 ± 8	94	575 ± 14	83
Control medium + DFO 10 μM	276 ± 22**	63	425 ± 35**	64	309 ± 24*	76	464 ± 7**	67
Control medium + DFO 100 μM	158 ± 5**	36	252 ± 17**	38	207 ± 9**	51	360 ± 25**	52
SAAD medium	1976 ± 49	100	2874 ± 32	100	1182 ± 67	100	2167 ± 118	100
SAAD medium + DFO 2 μM	1759 ± 33**	89	2529 ± 51**	88	1099 ± 36	93	2102 ± 44	97
SAAD medium + DFO 10 μM	1028 ± 11**	52	1523 ± 16**	53	567 ± 12**	48	1018 ± 33**	47
SAAD medium + DFO 100 μM	593 ± 10**	30	948 ± 7**	33	402 ± 43**	34	737 ± 56**	34

Cells were loaded with DCFH-DA and cultured in control or SAAD medium in the presence or absence of DFO for 5 or 10 min. Fluorescence of oxidized DCF was monitored at the excitation wavelength of 485 nm and the emission wavelength of 530 nm using a fluorescence plate reader. DCF oxidation was shown as an arbitrary unit. Data represent the mean ± SE from six separate experiments (significant as compared to the respective control or SAAD).
*$P < 0.05$; **$P < 0.01$.

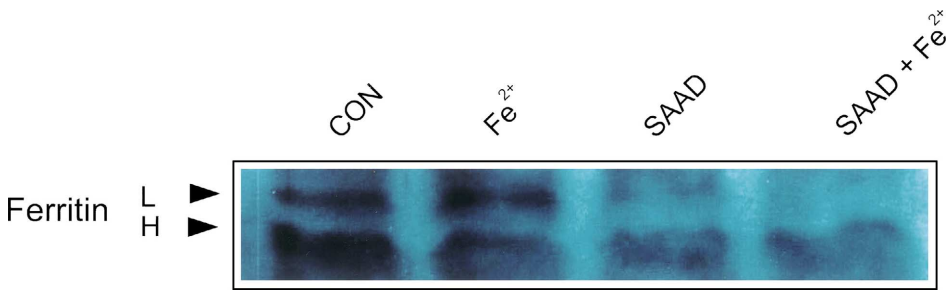

Fig. 6. Expression of ferritin light and heavy chain subunits in Raw264.7 cells. The representative immunoblot shows that Raw264.7 cells incubated in SAAD medium for 36 hr showed a marked decrease in the FLC protein level. Treatment of cells with 100 μM $FeSO_4$ for 12 hr following preincubation in SAAD medium for 24 hr failed to increase the FLC protein expression. Each lane was loaded with 80 μg of cell lysates. Data was confirmed by two repeated experiments.

however, markedly decreased in Raw264.7 cells exposed to SAAD medium for 36 hr (i.e. 15% of control) (Fig. 6). FLC protein was also decreased in Raw264.7 cells incubated in SAAD medium for 24 hr (i.e. 50% of control), whereas the protein level was not notably changed at 12 hr (data not shown). $FeSO_4$ (12 hr) failed to stimulate FLC expression in Raw264.7 cells under SAAD for 24 hr (Fig. 6). These results clearly showed that deficiency of sulfur amino acids down-regulated FLC protein.

3.6. Increase in the FLC mRNA level by SAAD

In contrast to the suppression of FLC protein, the FLC mRNA levels were increased by SAAD. Northern blot analysis revealed that constitutive FLC mRNA level was 4.2-fold greater in Raw264.7 cells than in Hepa1c1c7 (Fig. 7, Table 2). SAAD increased the FLC mRNA in both Hepa1c1c7 cells and Raw264.7 cells. The SAAD-inducible FLC mRNA level (24 hr) was also greater in Raw264.7 cells than that in Hepa1c1c7 cells (Fig. 7). A time-course study showed that the FLC mRNA level was increased 2- to 5-fold by SAAD in Hepa1c1c7 cells at 12–72 hr (Table 2). SAAD elevated the mRNA level in Raw264.7 cells at an early time point (i.e. a 3-fold increase at 12 hr), which gradually returned toward control at 72 hr (Table 2).

Table 2
The relative FLC mRNA levels in Hepa1c1c7 and Raw264.7 cells

Treatment	Relative FLC MrNA levels	
	Hepa1c1c7	Raw264.7
Control medium	1	4.2 ± 0.8*
SAAD medium		
12 hr	2.3 ± 0.2**	13 ± 2.3**,a
24 hr	3.6 ± 0.4**	7.9 ± 0.6**,a
48 hr	4.4 ± 0.3**	6.6 ± 1.4*
72 hr	4.7 ± 0.7**	3.9 ± 0.3**

The relative mRNA levels were assessed by scanning densitometry of Northern blots performed with total RNA fractions (30 μg each) prepared from cells in control or SAAD medium. Data represent the mean ± SE with three separate experiments. One way analysis of variance was used for comparisons of multiple group means followed by Newman–Keuls test.
[a] Significant as compared to Raw264.7 cells in control medium, $P < 0.05$ (control mRNA level in Hepa1c1c7 cells = 1).
* Significant as compared to the FLC mRNA in Hepa1c1c7 cells in control medium, $P < 0.05$.
** Significant as compared to the FLC mRNA in Hepa1c1c7 cells in control medium, $P < 0.01$.

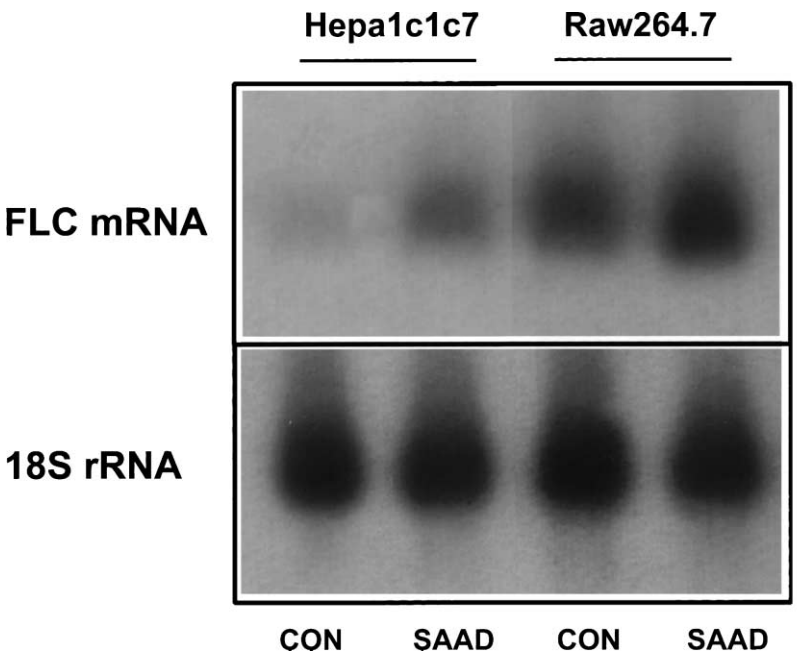

Fig. 7. The effects of SAAD on the FLC mRNA levels in Hepa1c1c7 and Raw264.7 cells. Representative Northern blot shows the relative mRNA levels in total RNA fractions (30 μg each) prepared from cells incubated in control or SAAD medium for 24 hr. The amount of RNA loaded in each lane was assessed by re-hybridization of the stripped membrane with a [^{32}P]-labeled probe for 18S rRNA.

We were then interested in whether increases in Ca^{2+} and free iron by SAAD were associated with the FLC mRNA increase. SAAD-inducible FLC mRNA expression was prevented by the presence of calcium or iron chelators (i.e. 100 μM EGTA or DFO) for 24 hr in Hepa1c1c7 cells, although the constitutive FLC mRNA level was less affected (Fig. 8). The FLC mRNA levels were decreased by the presence of calcium or iron chelators in Raw264.7 cells in control or SAAD medium. Phen Green SK, an iron chelator (20 μM, 24 hr) also inhibited the increase in FLC mRNA by SAAD in Hepa1c1c7 cells (data not shown). These results showed that increase in $[Ca^{2+}]_i$ and iron by SAAD enhanced the FLC mRNA expression.

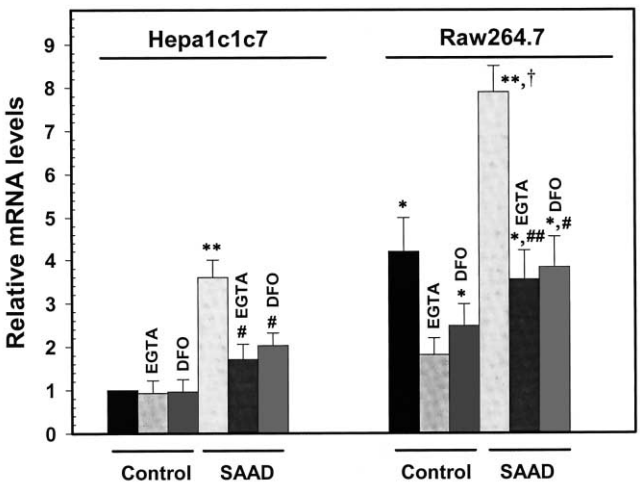

Fig. 8. The effects of EGTA and DFO on the increase in the FLC mRNA level by SAAD in Hepa1c1c7 and Raw264.7 cells. Northern blot analysis performed with total RNA fractions (30 μg each) prepared from cells incubated in SAAD medium for 24 hr with or without 100 μM EGTA or 100 μM DFO. Data represent the mean ± SE with three separate experiments. (significant as compared to Hepa1c1c7 in control medium, (*) $P < 0.05$, (**) $P < 0.01$; significant as compared to Raw264.7 in control medium, (†) $P < 0.01$; significant as compared to the respective cell line in SAAD medium, (#) $P < 0.05$, (##) $P < 0.01$) (Hepa1c1c7 in control medium = 1).

4. Discussion

In the present study, we investigated the effect of a decrease in GSH by SAAD on changes in $[Ca^{2+}]_i$ and free iron in two different cell types and the effect of SAAD on the FLC expression. We revealed that an increase in $[Ca^{2+}]_i$ was the initial event occurring in response to SAAD. The change in $[Ca^{2+}]_i$ by SAAD was relatively persistent, as compared with that by other stimuli (e.g. lysophosphatidic acid) [26]. Studies in this laboratory revealed that the increase in $[Ca^{2+}]_i$ by altered redox state was required for amplification of the downstream signals for the expression of responsive genes (Kang and Kim, unpublished data). The observation that either verapamil or thapsigargin inhibited an increase in $[Ca^{2+}]_i$ by SAAD raised the possibility that a change in the redox state by a decrease in GSH increased $[Ca^{2+}]_i$ as a consequence of extracellular Ca^{2+} influx and intracellular calcium release. Intracellular Ca^{2+} returned toward control after adding cystine or GSH to the cells. GSH is bidirectionally transported in hepatoma cells [27]. When we extended the incubation time, the fluorescence was below F_o (Fig. 2). This may result in part from the release of calcium from

the calcium bound to the dye before addition of SAAD medium.

In the present study, cells were treated with calcium channel blockers or Ca^{2+}-free medium to further assess whether the rise in $[Ca^{2+}]_i$ affected iron release. An increase in $[Ca^{2+}]_i$ appeared to be coupled with that in free iron release. Fluorescence microscopic analysis revealed that an increase in $[Ca^{2+}]_i$ led to an increase in free iron particularly in dye-loaded Hepa1c1c7 cells. The cell-type specific increase in $[Ca^{2+}]_i$ was consistent with the release of iron. Inhibition of the iron release from Phen Green SK-bound iron by the inhibition of Ca^{2+} mobilization supported the hypothesis that increase in $[Ca^{2+}]_i$ by change in the redox state indeed affects cellular iron release.

A previous study from this laboratory showed that SAAD increased DCF oxidation in H4IIE rat hepatoma cells [3]. In the current study, deficiency of sulfur amino acids rapidly increased prooxidant production in both Hepa1c1c7 cells and Raw264.7 cells. Inhibition of Ca^{2+} mobilization by verapamil or thapsigargin or by Ca^{2+}-free condition significantly inhibited DCF oxidation. Hence, cellular Ca^{2+} contributed to prooxidant production. DFO, a strong iron chelator, was more active in preventing prooxidant production, which was in agreement with the previous reports [28,29–31]. Prooxidant production was almost completely inhibited by DFO in both Hepa1c1c7 cells and Raw264.7 cells in control or SAAD medium, indicating that iron was directly responsible for prooxidant production. The present study demonstrated that an increase in $[Ca^{2+}]_i$ was a prerequisite for the release of iron and subsequent oxidative stress. The extent of prooxidant production is not necessarily proportional to the concentration of free iron because free iron serves as a catalyst for the formation of peroxides and may also further change $[Ca^{2+}]_i$. Other antioxidant defense system would also contribute to the change.

Ferritin as an iron storage protein takes and releases iron inside cells. Because the bulk of iron is stored as a core of $Fe(OH)_3$ in ferritin, ferritin serves as a potentially harmful iron donor as well as an effective antioxidant. Although, FLC serves as an iron donor, the mechanistic basis for the iron release has not been clarified yet. Ferritin is synthesized in response to oxidative stress (e.g. in the liver). Ferritin H and L mRNA levels were also increased via transcriptional activation. An increase in cellular free iron accompanied the transcription of ferritin mRNA and its translation. In the present study, we showed for the first time that the expression of FLC was much greater in Raw264.7 cells than that in Hepa1c1c7 cells. This supported the notion that Raw264.7 cells are more resistant to iron-mediated oxidative stress. SAAD increased intracellular iron to a greater extent in Hepa1c1c7 cells than in Raw264.7 cells. This might result from the lower level of constitutive FLC in hepatocytes. Oxidative stress was inversely correlated with the constitutive expression of ferritin protein.

Studies have shown that an increase in iron availability stimulates both gene transcription and ferritin synthesis [14,22]. SAAD increased a free iron content in both Hepa1c1c7 and Raw264.7 cells. FLC protein could be detected in Raw264.7 cells, but not in Hepa1c1c7 cells. This was consistent with the lower fluorescence intensity of chelatable iron in Raw264.7 cells, which in conjunction with a high level of ferritin may be associated with a greater resistance of macrophages to oxidative stress.

In the current study, we showed that SAAD down-regulated FLC expression. We demonstrated for the first time that deficiency of sulfur amino acids down-regulated FLC. Cysteine residues of the proteins binding with iron-response element (IRE) have been shown to be involved in the translational control of FLC [32,33]. Hence, suppression of FLC protein by SAAD might result from a decrease in the translational efficiency of ferritin mRNA as a result of a decrease in IRE binding proteins and/or changes in other components involved in translation. Utilization of the mRNA and mRNA turnover (stability) would also affect synthesis of the protein. This raised the notion that cysteine is critical for the control of FLC protein expression, especially in the state of oxidative stress. Phorone an agent depleting sulfhydryls, however, increased translation of the ferritin mRNA following GSH depletion [8]. FLC expression in response to oxidative stress accompanying deficiency of sulfur amino acids appeared to differ from that caused by chemical-induced oxidative stresses (e.g. difference in the time-course of cellular GSH content) [8].

A decrease in FLC protein expression may also result from accelerated degradation due to deficiency of sulfur amino acids. Previous studies have shown that deprivation of total amino acids or serum led to autophagocytosis of cytosolic ferritin and decreased the intracellular GSH concentration [25]. In the study, DFO-available pool of iron increased although, the total iron content was not affected. Oxidative stress would induce degradation of ferritin, which further increases an iron pool inside cells. In particular, sulfur amino acids may also be involved in controlling the autophagic release of free iron from ferritin. Hence, both inhibition of FLC protein synthesis and degradation of FLC protein would be responsible for the change in FLC expression in cells under SAAD.

Although, SAAD failed to induce FLC protein, the transcription of the *FLC* gene was not perturbed by SAAD, as evidenced by the increase in FLC mRNA in Northern blot analysis. This result is clearly different from that by oxidizing agents such as diamide and organic hydroperoxides [33,34]. Hence, the cell culture model in SAAD medium gives us the opportunity to assess the role of GSH depletion in the expression of FLC. Increase in the FLC mRNA level by oxidative stress might result from a compensatory increase in the gene expression and/or a change in the mRNA stability. SAAD increased the FLC mRNA level in Hepa1c1c7 cells and to a greater extent in Raw264.7 cells. We provided strong evidence that

chelation of either Ca^{2+} or free iron prevented the increase in FLC mRNA by SAAD. This supports the hypothesis that $[Ca^{2+}]_i$ increase stimulates release of free iron from ferritin, which may serve as a stimulator of the FLC gene expression. A study in our laboratory showed that the rise in $[Ca^{2+}]_i$ was controlled by phosphoinositide 3-kinase (Kang and Kim, unpublished data). Also, this enzyme was responsible for prooxidant production, as evidenced by the complete blocking of DCF formation in the presence of phosphoinositide 3-kinase inhibitors. Hence, the phosphoinositide 3-kinase is one of the enzymes, which sense the altered redox state. Exposure of cells to SAAD medium causes rapid change in $[Ca^{2+}]_i$ and activates the cellular signaling pathways, at early times (e.g. 10 min to 3 hr) [5], (Kang and Kim, unpublished data). In our preliminary study, wortmannin or LY294002, phosphoinositide 3-kinase inhibitors, partly inhibited the increase in FLC mRNA by SAAD in Raw264.7 cells. These results support the role of $[Ca^{2+}]_i$ in the FLC gene expression. The essential role of phosphoinositide 3-kinase on other antioxidant gene expression (e.g. glutathione-*S*-transferases) has also been demonstrated in this laboratory [3]. The inhibitors of MAP kinases, PD98059 and SB203580, were ineffective in blocking the FLC gene expression by SAAD (Kim and Kim, unpublished data). An additional study showed that cells exposed to SAAD medium for 48–72 hr began to die with the release of cytochrome *c* (Kang and Kim, unpublished data), which may be associated with accumulation of free iron and oxidative stress.

In summary, the results of this study demonstrated that deficiency of sulfur amino acids increased susceptibility of cells to oxidative stress as a consequence of increases in $[Ca^{2+}]_i$ and free iron. This might also be associated with the suppression of FLC protein expression in spite of the increase in the FLC mRNA.

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