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Identification of a mechanism of iron uptake by cells which is stimulated by hydroxyl radicals generated via the iron-catalysed Haber–Weiss reaction

D.R. Richardson [a,b,*], P. Ponka [a,b]

[a] *Lady Davis Institute for Medical Research of the Sir-Mortimer B. Davis-Jewish General Hospital, 3755 Chemin de la Cote-Ste-Catherine, Montreal, Qué., H3T 1E2, Canada*

[b] *Departments of Physiology and Medicine, McGill University, Montreal, Qué., Canada*

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Abstract

Recent studies have demonstrated that preincubation of SK-Mel-28 melanoma cells with ferric ammonium citrate (FAC) resulted in marked stimulation of ^{59}Fe uptake from ^{59}Fe-^{125}I-transferrin (Tf), but only at Tf concentrations above that required for saturation of the Tf receptor (Richardson and Baker (1992) J. Biol. Chem. 267, 13972–13979). The mechanism responsible for this stimulation was unknown and is the subject of the present report. Preincubation of cells with FAC (25 μg/ml), followed by a 2 h incubation with ^{59}Fe-^{125}I-Tf (0.1 mg/ml; 1.25 μM), resulted in temperature-dependent ^{59}Fe uptake to approx. 200% of the control value. Furthermore, the effect was not specific for melanoma cells and was also observed in other normal and neoplastic cells. Preincubation of melanoma cells with FAC also stimulated ^{59}Fe uptake from ^{59}Fe-citrate, but to a far greater extent than that observed with ^{59}Fe-^{125}I-Tf (viz., > 20-fold that seen for the control). Interestingly, neither receptor-mediated endocytosis nor the postulated diferric Tf reductase were involved in the FAC-activated Fe uptake process from Tf. However, the addition of free radical scavengers to FAC such as catalase, superoxide dismutase, ceruloplasmin, Hepes, mannitol and high concentrations of BSA or ascorbate, markedly depressed FAC-activated ^{59}Fe uptake from ^{59}Fe-^{125}I-Tf and ^{59}Fe-citrate. These agents when added to control cells had no effect on ^{59}Fe uptake. The addition of superoxide generating agents and hydrogen peroxide to minimum essential medium (MEM) containing FAC but not to MEM alone, also stimulated ^{59}Fe uptake. These data suggest that the initial activation of the FAC-stimulated Fe uptake system was caused by the production of hydroxyl radicals via the Fe-catalysed Haber–Weiss reaction. We propose that this Fe uptake process represents an important cellular defense mechanism against oxidant stress generated in the presence of low-molecular-weight Fe complexes.

Keywords: Haber–Weiss reaction; Iron uptake; Hydroxyl radical; Iron; Transferrin

1. Introduction

Iron (Fe) containing proteins catalyse key reactions involving energy metabolism, respiration and DNA synthesis [1]. Donation of Fe to cells occurs following the binding of Tf to the transferrin receptor (TfR) on the cell membrane. The Tf-TfR complex is then internalised by receptor-mediated endocytosis (RME), and Fe is released from Tf by a process involving endosomal acidification and reduction [2–4]. In addition to this well characterised mechanism, several investigators have also reported an alternate process mediated by a surface-bound NADH:ferricyanide oxidoreductase [5–9]. However, the role of an oxidoreductase (diferric Tf reductase) in Fe uptake from Tf is poorly defined, and further characterisation of this process is required, as the results remain controversial [10,11].

Apart from Tf-bound Fe uptake, mammalian cells also possess a high capacity, membrane-bound, Fe-transport system capable of efficiently obtaining Fe from small molecular weight (M_r) complexes [12–16]. Incubation of a wide variety of cell types with the Fe complex, ferric ammonium citrate (FAC), results in the activation of a

Abbreviations: DFO, desferrioxamine; FAC, ferric ammonium citrate; gPR, gram of protein; MB, methylene blue; MEM, minimum essential medium; RME, receptor-mediated endocytosis; SOD, superoxide dismutase; Tf, transferrin; TfR, transferrin receptor.

* Corresponding author. Tel.: +1 (514) 3408260; fax: +1 (514) 3407502.

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membrane-bound Fe transport system [17] that may be at least partly responsible for the Fe uptake observed in hypotransferrinemia and Fe overload disease [15,18].

Our previous studies investigating the Fe uptake processes of the human melanoma cell line, SK-MEL-28, demonstrated two separate Fe uptake mechanisms from Tf [19]. The first process was consistent with RME which saturated at a Tf concentration of 0.01 mg/ml (0.125 μM), and was mediated by a single high affinity Tf binding site. The second Fe uptake process was saturable at much higher Tf concentrations (0.3–0.5 mg/ml), and was consistent with low affinity adsorptive pinocytosis [20]. Melanotransferrin [21] appeared to play little role in Fe uptake from Tf [16,19,22–24]. Further work demonstrated that preincubation of melanoma cells with FAC resulted in down-regulation of the specific Tf-binding site [25]. Paradoxically, FAC also markedly stimulated the rate of Fe uptake from Tf at Tf concentrations above that required for saturation of the TfR, without increasing the rate of Tf uptake [25]. It was hypothesized that FAC may be acting to stimulate the postulated diferric Tf reductase by acting as an electron acceptor, enhancing electron transport across the membrane and increasing Fe uptake from Tf. It was relevant to investigate this hypothesis as it may have important consequences in defining the relative roles of the two main mechanisms of Fe uptake from Tf, namely RME and the alternate process mediated by an oxidoreductase. Additionally, as described above, previous work in other cell types has demonstrated that FAC can also stimulate the uptake of Fe from small M_r Fe complexes. However, the mechanism responsible for activating this Fe uptake system was not established [17], and hence, it was also of interest to examine the relationship between FAC stimulated Fe uptake from Tf and small M_r Fe complexes.

The present study demonstrates that FAC stimulates ^{59}Fe uptake from ^{59}Fe-^{125}I-Tf and ^{59}Fe-citrate by a novel Fe transport mechanism which does not involve the TfR or a diferric Tf reductase. This Fe uptake system is activated by hydroxyl radicals produced via the Fe-catalysed Haber–Weiss reaction, and it is suggested that this process may be an important protective mechanism to guard against oxidant stress generated in the presence of small M_r Fe complexes in pathophysiological states such as Fe overload disease.

2. Materials and methods

2.1. Reagents

All reagents were obtained from the same suppliers as described previously [16,23,25].

2.2. Cells

The SK-Mel-28 human melanoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and Chinese hamster ovary cells (wild type and variant A (Va) mutant deficient in the TfR) were kind gifts from Dr. F.R. Maxfield [26]. Cells were grown and subcultured as described by Richardson and Baker [19].

2.3. Protein purification and labelling

Human apoTf was prepared and labelled with ^{59}Fe and ^{125}I as described [19]. Apolactoferrin (ALF) was labelled with ^{56}Fe using the same technique described above for apoTf. Monoclonal antibodies (MoAb) specific for the human TfR (42/6) and human Tf (HTF-14) were kind gifts from Dr. I. Trowbridge (Salk Institute, San Diego, CA) and Dr. A. Hradilek (Institute of Hematology and Blood Transfusion, Prague), respectively.

2.4. Experimental procedure-uptake of iron and transferrin after exposure to ferric ammonium citrate

2.4.1. Preincubation procedure

Cells were preincubated with MEM (control) or MEM containing FAC (25 μg/ml;[Fe] = 4 μg/ml) using three 45 min incubations at 37°C [24], as this procedure was found to be most effective at stimulating ^{59}Fe uptake from ^{59}Fe-^{125}I-Tf [24]. FAC at concentrations up to 150 μg/ml ([Fe] = 26 μg/ml) had no adverse effects on cells, as judged by LDH release, uptake of ^{3}H-leucine, trypan blue staining and cellular adherence. Furthermore, FAC has been commonly used at this concentration to load cells with Fe with no deleterious consequences [27–29].

2.4.2. Labelling procedure

After the preincubation procedure, the medium was replaced with medium containing ^{59}Fe-^{125}I-Tf ([Tf] = 0.1 mg/ml; [Fe] = 2.5 μM) or ^{59}Fe-citrate (1:100; [Fe] = 2.5 μM) which was then incubated with the cells for 2 h. It should be noted that a 1:100 molar ratio of ^{59}Fe to citrate was used to prevent polymerisation [30,31]. Previous studies investigating low-M_r Fe uptake have used nitrilotriacetic acid (NTA) to chelate ^{59}Fe [15,17,32]. However, NTA is a non-physiologically relevant ligand, and in the present investigation ^{59}Fe-citrate was used as this is the prevailing form of non-Tf-bound Fe found in the serum of patients with Fe-overload [33]. In addition, earlier studies using melanoma cells demonstrated that ^{59}Fe donated to the cell from citrate-^{59}Fe is efficiently internalised and incorporated into ferritin and other compartments [16].

In some experiments various agents were added to the preincubation or labelling solutions to assess their effects on Fe uptake from Tf or Fe-citrate. When alteration to the standard procedures are implemented, they are described in the text. After incubation with ^{59}Fe-^{125}I-Tf or ^{59}Fe-citrate, the medium was removed and the cells washed 4-times with ice-cold balanced salt solution. Pronase (1 mg/ml) was then incubated with the cells for 30 min at 4°C to

separate internalised from membrane-bound ^{59}Fe and ^{125}I-Tf, as described [19,25,34].

Results were calculated as moles of Fe or Tf per gram of protein (gPR) and as a percentage of the control and are expressed as the mean or mean ± S.D. Each determination was derived from 1 confluent petri dish containing 5×10^6 cells, and this was equivalent to about 0.45 mg of protein via the bicinchoninic acid protein assay procedure [19].

3. Results

3.1. Effect of ferric ammonium citrate concentration on iron uptake from transferrin

The ^{59}Fe uptake from Tf increased as the FAC concentration increased, and then plateaued at FAC concentrations from 25–75 μg/ml, where ^{59}Fe uptake varied from 194 ± 4% (4 determinations) to 184 ± 4% (4 determinations) of the control value, respectively (Fig. 1). Increasing the FAC concentration to more than 75 μg/ml decreased ^{59}Fe uptake from Tf, and at a FAC concentration of 150 μg/ml, ^{59}Fe uptake decreased to approx. 62 ± 1% (4 determinations) of the control (Fig. 1). At present we do not understand why there is a decrease in internalised Fe uptake at high FAC concentrations. However, it should be noted that the decrease in Fe uptake was not due to toxicity associated with FAC, as there was no change in cellular viability or adherence of the cells to the culture substratum. As a FAC concentration of 25 μg/ml was the minimum required to obtain maximum stimulation of ^{59}Fe uptake (Fig. 1), this concentration was used in all other experiments.

Exposure of cells to FAC for only 5 min was sufficient to produce enhanced Fe uptake after a 2 h incubation with Tf (data not shown), suggesting that de novo synthesis of a transporter did not occur. Ferric ammonium citrate-activated Fe uptake is not unique to SK-Mel-28 melanoma cells, and was seen in all cells examined, including Chinese hamster ovary (CHO) cells (Va mutant and wild-type), human baby foreskin fibroblasts (provided by Dr. R. Germinario, Lady Davis Institute, Montreal) and a human embryonic kidney cell line (ATCC CRL 1573). However, as the Fe metabolism of the melanoma cell is well characterised [16,19,20,22–25,35], and since our initial report on the FAC-activated mechanism was performed using these cells [25], we decided to further characterise this process using the SK-Mel-28 melanoma cell line.

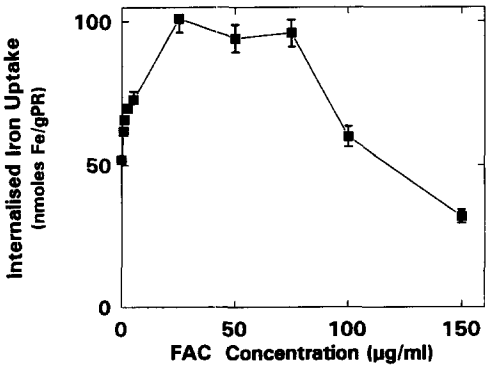

Fig. 1. The effect of ferric ammonium citrate (FAC) concentration (0.5–150 μg/ml) on internalised ^{59}Fe uptake from ^{59}Fe-^{125}I-Tf (0.1 mg/ml) by melanoma cells. Results are the mean ± S.D. of 3–5 determinations in a typical experiment of 2 experiments performed.

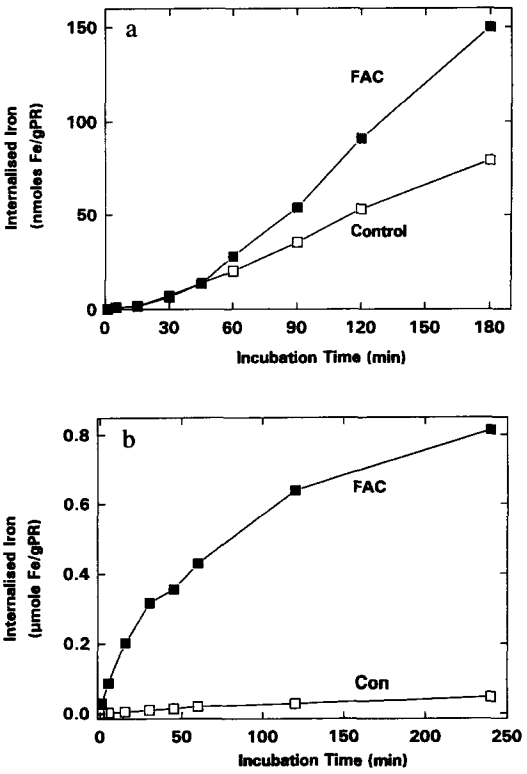

Fig. 2. The effect of incubation time with (a) ^{59}Fe-^{125}I-Tf (0.1 mg/ml; [Fe] = 2.5 μM) or (b) ^{59}Fe-citrate ([Fe] = 2.5 μM; molar ratio of ^{59}Fe:citrate = 1:100) on internalised ^{59}Fe uptake by melanoma cells preincubated with MEM or MEM containing ferric ammonium citrate (FAC). Results are the means of 2 determinations in a typical experiment of 3 experiments performed. The variation between duplicates was less than 5%.

3.2. Effect of incubation time with ^{59}Fe-^{125}I-transferrin or citrate-^{59}Fe complexes on ferric ammonium citrate-activated iron uptake

To examine the effect of incubation time with ^{59}Fe-^{125}I-Tf (0.1 mg/ml; [Fe] = 2.5 μM) on FAC-activated ^{59}Fe uptake, cells were exposed to MEM or MEM containing FAC as described, and then incubated with Tf for up to 3 h. Interestingly, FAC-activated ^{59}Fe uptake was only observed after 60 min of incubation with Tf (Fig. 2a). In contrast, when melanoma cells were treated with FAC and then exposed to ^{59}Fe-citrate ([Fe] = 2.5 μM), stimulation of ^{59}Fe uptake was observed after only 1 min of incubation (Fig. 2b). The extent of stimulation with ^{59}Fe-citrate as an

Fe donor was far greater than that obtained with ^{59}Fe-Tf, viz. after a 2 h incubation with ^{59}Fe-citrate, FAC stimulated Fe uptake was 23-fold greater than that seen for the control (Fig. 2b). To investigate the mechanism responsible for the FAC-activated Fe uptake process, experiments were designed to examine whether ^{59}Fe uptake occurred by the diferric Tf reductase or RME.

3.3. The role of the diferric transferrin reductase (oxidoreductase) in ferric ammonium citrate-activated iron uptake

Considering our results demonstrating the stimulatory effect of FAC on Fe uptake from Tf ([25] and this report), we hypothesized that FAC could be acting as an oxidant to enhance oxidoreductase activity [36,37], resulting in an increase in Fe uptake from Tf. To test this hypothesis other oxidants were investigated. Indeed, Hexammineruthenium(III) chloride and 1,2-naphthoquinone-4-sulfonate both stimulate oxidoreductase activity [38], and in the present study also increased Fe uptake from Tf, although to a lesser extent than FAC (Table 1). However, other oxidants such as indigotetrasulfonate, ferricyanide [38] and unlabelled diferric Tf [39], had no stimulatory effect (Table 1). In addition, amiloride, vanadate and chloroquine are all inhibitors of oxidoreductase activity [5,7,39,40]. However, when amiloride (0.2 mM) was added to ^{59}Fe-^{125}I-Tf, it had little effect on ^{59}Fe uptake by control cells or cells preincubated with FAC (Table 2), whereas vanadate and chloroquine decreased Fe uptake by control cells but had no effect on FAC-activated Fe uptake (data not shown). Collectively these data suggest that an oxidoreductase may not be responsible for FAC-activated ^{59}Fe uptake from Tf.

Table 2
Effect of monoclonal antibody, 42/6, and inhibitors of receptor-mediated endocytosis and oxidoreductase activity on iron uptake by the ferric ammonium citrate (FAC)-activated process

Preincubation Incubated with:	Labelling Incubated with:	Internalised iron (% control)
MEM (control)	Tf	100
FAC	Tf	210
MEM	Tf + Amiloride (0.2 mM) [a]	93
FAC	Tf + Amiloride (0.2 mM) [a]	192
MEM	Tf + NH_4Cl (15 mM)	15
FAC	Tf + NH_4Cl (15 mM)	132
MEM	Tf + 42/6 (100 μg/ml)	35
FAC	Tf + 42/6 (100 μg/ml)	161

Cells were preincubated with MEM or MEM-containing FAC (25 μg/ml) for three 45 min incubations at 37°C. This medium was removed and the cells then labelled for 2 h at 37°C with ^{59}Fe-^{125}I-Tf (0.1 mg/ml) in the presence and absence of the inhibitors.
[a] Amiloride at concentrations up to 2 mM had a similar effect.
Results are mean values of 2–3 determinations from a typical experiment of 3 experiments performed. The variation between duplicates or triplicates was less than 5%.

3.4. The role of receptor-mediated endocytosis in ferric ammonium citrate-activated iron uptake

Ammonium chloride (NH_4Cl; 15 mM) is an inhibitor of endosomal acidification [41], and when incubated with Tf this agent reduced ^{59}Fe uptake to 15% of the control in cells preincubated with MEM (Table 2). When NH_4Cl was included with ^{59}Fe-Tf after the initial preincubation with FAC, there was also a decrease in ^{59}Fe uptake (Table 2). However, this decrease can be accounted for by the inhibition of Fe uptake via the endocytotic processes, and hence, there has been no decrease in Fe uptake via the FAC-

Table 1
The effect of preincubation with ferric ammonium citrate (FAC) or other oxidants on internalised iron uptake from transferrin by SK-MEL-28 melanoma cells

Oxidant	% Control	
	Internalised Fe	Internalised Tf
MEM (Control)	100 ± 1 [a]	100 ± 3
FAC (25 μg/ml)	205 ± 6	105 ± 5
Citrate (25 μg/ml)	98 ± 3	102 ± 1
Unlabelled diferric Tf (2.5 μM)	88 [b]	95
Hexammineruthenium(III) chloride (0.1 mM)	145 ± 4	104 ± 2
Hexammineruthenium(III) chloride (0.5 mM)	162 ± 7	105 ± 5
1,2-Naphthoquinone-4-sulfonate (0.1 mM)	129 ± 6	103 ± 1
Indigotetrasulfonate (0.1 mM)	95 ± 3	102 ± 3
Ferricyanide (0.5 mM)	103 ± 3	96 ± 3
Ferrocyanide (0.5 mM)	105 ± 5	102 ± 4
Cytochrome *c* (1 mg/ml)	94 ± 3	98 ± 3

Cells were preincubated with the oxidants using three 45 min incubations at 37°C. The medium was then removed and replaced with medium containing ^{59}Fe-^{125}I-Tf (0.1 mg/ml) which was incubated with the cells for 2 h at 37°C.
[a] Results are mean ± S.D. (3 determinations) from a typical experiment of 2–3 experiments performed.
[b] Results are mean values of duplicate determinations from a typical experiment of 2 experiments performed. The variation between duplicates was less than 5%.

activated process (Table 2). To further test whether FAC-activated Fe uptake was mediated by the TfR, the effect of MoAb 42/6 which prevents the binding of Tf to the TfR [42] was also examined. When this MoAb (100 μg/ml) was added together with ^{59}Fe-^{125}I-Tf, it markedly reduced ^{59}Fe uptake to 35% of the control value in cells preincubated with MEM (Table 2). On the other hand, for cells preincubated with FAC, the decrease in ^{59}Fe uptake seen after incubation with Tf and 42/6 (Table 2), can be accounted for by the inhibition of the endocytotic process, and hence, this MoAb had no significant effect on the FAC-activated mechanism.

Further evidence that the TfR is not involved in FAC-activated Fe uptake was obtained by examining the effect of FAC on ^{59}Fe uptake from ^{59}Fe-^{125}I-Tf by wild-type (WT) Chinese hamster ovary (CHO) cells expressing the TfR and mutant (Va) CHO cells deficient in TfR expression [26,43]. Comparison of internalised ^{59}Fe uptake by the WT and Va CHO cells preincubated with MEM only (Table 3), demonstrated that Va cells can obtain little Fe from Tf. However, for both cell types, preincubation with FAC effectively stimulated Fe uptake (Table 3). After exposure to FAC, internalised ^{59}Fe uptake from Tf was equivalent to 159% and 2400% of the control values (i.e., MEM) for the WT and Va cell lines, respectively (Table 3). Interestingly, a large increase in membrane ^{59}Fe uptake by both wild-type and mutant CHO cells also occurred after activation with FAC, which may indicate the presence of an membrane-bound Fe binding component (Table 3). Further, there was also an increase in the internalised molar ratio of Fe:Tf, and this was particularly marked for Va cells, where it increased from 8 (control) to 98 (FAC-activated). In addition, for Va cells, the percentage of the total ^{59}Fe which was internalised after exposure to FAC increased from 30% to 74%, whereas there was no increase in the percentage of ^{125}I-Tf internalised (Table 3). These data, together with that described above, clearly demonstrate that the TfR is not required for FAC-activated Fe uptake.

To further characterise FAC-activated Fe uptake from Tf, the effect of temperature was also investigated. When cells were preincubated with FAC at 4°C instead of 37°C, and then labelled with Tf at 37°C, FAC at 4°C was still able to stimulate ^{59}Fe uptake from ^{59}Fe-Tf to 153% of the control. However, if the cells were preincubated with FAC at 37°C as usual, and then labelled with Tf at 4°C, there was no uptake of ^{59}Fe from ^{59}Fe-Tf, suggesting that the cell must be metabolically active to remove Fe from Tf.

3.5. Does the ferric ammonium citrate-activated process remove iron from transferrin at the cell surface and does it require direct interaction of transferrin with the plasma membrane ?

To examine the mechanism of Fe release from Tf by the FAC-activated process, cells preincubated with MEM or FAC were then labelled with ^{59}Fe-^{125}I-Tf (0.1 mg/ml) containing apolactoferrin (ALF; 0.3 mg/ml) or diferric lactoferrin (DLF; 0.3 mg/ml), neither of which binds to the TfR [44]. When ALF was added together with Tf, and then incubated with cells previously exposed to FAC, ALF completely prevented FAC-activated Fe uptake (Table 4). ALF had no effect on Fe uptake from Tf in control cells preincubated with MEM only. In contrast, DLF had little effect on ^{59}Fe uptake in cells preincubated with FAC or MEM (Table 4). These results suggest that after activation with FAC, Fe was released from Tf at the outer cell membrane or in solution and then chelated by ALF.

Previous work has demonstrated that melanoma cells have only one class of Tf-binding sites [19,20], which as shown above, are not involved in FAC-activated Fe uptake. In addition, FAC-activated Fe uptake was not due to an increase in the activity of the adsorptive pinocytosis pathway [20], as there was no increase in the rate of

Table 3
Effect of preincubation with ferric ammonium citrate (FAC) on iron uptake from ^{59}Fe-^{125}I-transferrin (0.1 mg/ml) by wild-type and variant A (Va) Chinese hamster ovary cells deficient in the transferrin receptor

	Intern.Fe (% Control)	Intern. Fe:Tf	Memb. Fe (% Control)	%Fe Intern.	%Tf Intern.
Control Wild type	100 ± 4 [a] (42.3)	59 ± 1	100 ± 6 (4.4)	91 ± 1	29 ± 2
FAC Wild type	159 ± 3 (67.1)	74 ± 1	220 ± 13 (9.7)	87 ± 1	30 ± 3
Control Va	100 ± 6 (1.2)	8 ± 1	100 ± 9 (2.8)	30 ± 1	9 ± 1
FAC Va	2400 ± 65 (28.8)	98 ± 6	357 ± 34 (10.0)	74 ± 2	10 ± 1

Cells were preincubated with MEM (control) or MEM containing FAC (25 μg/ml) for three 45 min incubations at 37°C. This medium was removed and the cells then labelled with ^{59}Fe-^{125}I-Tf (0.1 mg/ml) for 2 h at 37°C.
Results are mean ± S.D. (4–5 determinations) from a typical experiment of two experiments performed.
[a] Results in parentheses represent Fe uptake expressed as nmole Fe/gPR.

Table 4
The effect of apolactoferrin (ALF) and diferric lactoferrin (DLF) on internalised ^{59}Fe uptake from ^{59}Fe-^{125}I-Tf by melanoma cells

Preincubation Incubated with:	Labelling Incubated with:	Internalised iron (% Control)
MEM (control)	Tf	100
FAC + MEM	Tf	244
MEM	Tf + ALF	94
FAC + MEM	Tf + ALF	87
MEM	Tf + DLF	91
FAC + MEM	Tf + DLF	235

Cells were preincubated with MEM (control) or MEM-containing FAC (25 μg/ml) for three 45 min incubations. This medium was removed and the cells then labelled for 2 h at 37°C with ^{59}Fe-^{125}I-Tf (0.1 mg/ml) only, or ^{59}Fe-^{125}I-Tf (0.1 mg/ml) and ALF (0.3 mg/ml), or ^{59}Fe-^{125}I-Tf (0.1 mg/ml) and DLF (0.3 mg/ml). The results are the means of 2 determinations in a typical experiment of 3 experiments performed. The variation between duplicates was less than 5%.

non-specific Tf uptake [25]. Moreover, FAC-activated Fe uptake was only observed at Tf concentrations above that required for saturation of the TfR (i.e., > 0.01 mg/ml; [25]). These data together with the fact that non-specific adsorption increases linearly with Tf concentration after saturation of the TfR [20], could indicate that FAC-activated Fe uptake is mediated by non-specific adsorption of Tf to the cell membrane.

To investigate whether specific-binding of ^{59}Fe-^{125}I-Tf to the cell membrane was required for FAC-activated Fe uptake, the effect of the anti-Tf MoAb, HTF-14 (HTF), was examined. Previous studies by Hradilek and Neuwirt [45] have demonstrated that treatment of ^{59}Fe-^{125}I-Tf with HTF results in an immune complex which prevents Tf from interacting with the specific TfR and also inhibits Fe uptake. In the present study, HTF (0.1 mg/ml) was incubated for 3 h at 37°C with ^{59}Fe-^{125}I-Tf (0.1 mg/ml) to produce ^{59}Fe-^{125}I-Tf-HTF complexes (Tf-HTf). Preincubation of cells with MEM followed by incubation with Tf-HTF decreased ^{59}Fe uptake to 8% of that found when control cells were incubated with ^{59}Fe-^{125}I-Tf. However, when cells were preincubated with FAC and then exposed to Tf-HTF, ^{59}Fe uptake was 53% of that found when these cells were incubated with ^{59}Fe-^{125}I-Tf alone. This HTF-mediated decrease in ^{59}Fe uptake observed in FAC-treated cells can be accounted for by the inhibition of specific RME and the non-specific adsorptive pinocytosis process in these cells [20]. Hence, the FAC-activated process could still efficiently remove Fe from Tf, even though the HTF-Tf immune complex could prevent the specific interaction of Tf with the cell membrane. These results may suggest that the Tf-HTF immune complex keeps Tf out of contact with the cell surface and that Fe uptake mediated via the FAC-activated process occurs in solution. Alternatively, the FAC-stimulated mechanism may only remove Fe from Tf when the HTf-Tf immune complex becomes non-specifically adsorbed to the cell membrane.

Evidence that non-specific adsorption of Tf to the cell surface is required for FAC-activated Fe uptake is suggested by the fact that when BSA (70 mg/ml) was added with ^{59}Fe-^{125}I-Tf (0.1 mg/ml), it completely inhibited FAC-activated Fe uptake, while having no effect on Fe uptake by control cells (data not shown). Concentrations of BSA less than 70 mg/ml were far less effective at preventing internalised Fe uptake. For example, BSA added to Tf at concentrations from 1–5 mg/ml had no effect on FAC-activated Fe uptake, whereas BSA at a concentration of 40 mg/ml resulted in only a 46% decrease in Fe uptake by the FAC-stimulated process. It is also important to note that FAC-activated Fe uptake is observed only after 60 min of incubation with Tf (Fig. 2a), whereas stimulation from Fe-citrate was evident after only 1 min of incubation with this complex (Fig. 2b). Since it is generally known that non-specific adsorption increases with increasing incubation time, these results suggest that FAC-stimulated Fe uptake from Tf is a process mediated by non-specific adsorption to the cell membrane. On the other hand, ^{59}Fe uptake from citrate may be the result of direct interaction with the same transport mechanism.

3.6. The effect of free radical scavengers on iron uptake

To examine whether free radicals played a role in FAC-activated Fe uptake, catalase and superoxide dismutase (Table 5) were added to FAC. These enzymes were found to be highly effective at preventing FAC-activated Fe uptake from Tf. In contrast, BSA added as a protein control at an equivalent concentration (0.05 mg/ml) had no effect on Fe uptake (Table 5). Other radical scavengers such as Hepes [46], desferrioxamine (DFO), mannitol or high concentrations of ascorbic acid (5 mM; [47]) or BSA

Table 5
The internalised iron uptake by SK-MEL-28 cells after the addition of catalase (1000 U/ml) and/or superoxide dismutase (SOD; 1000 U/ml) to the preincubation solutions

Preincubation Incubated with:	Labelling Incubated with:	Internalised Fe (% Control)
MEM (control)	Tf	100 ± 10
FAC	Tf	181 ± 4
BSA [a] + FAC	Tf	176 ± 5
Catalase + MEM	Tf	107 ± 3
Catalase + FAC	Tf	95 ± 1
SOD + MEM	Tf	100 ± 8
SOD + FAC	Tf	124 ± 4
Catalase + SOD + FAC	Tf	82 ± 8

Cells were preincubated with MEM or FAC (25 μg/ml) in the presence and absence of catalase or SOD or both for three 45 min incubations at 37°C. This medium was removed and the cells then labelled with ^{59}Fe-^{125}I-Tf (0.1 mg/ml) for 2 h at 37°C. Results are mean ± S.D. (3 determinations) from a typical experiment of 2 experiments performed.
[a] BSA was used as a protein control at an equivalent concentration to catalase and SOD, and at the low concentration used (0.5 mg/ml), did not inhibit FAC-stimulated ^{59}Fe uptake from ^{59}Fe-^{125}I-Tf.

Table 6
The internalised iron uptake by SK-MEL-28 cells after the addition of ceruloplasmin, ascorbate, desferrioxamine, the buffer Hepes, mannitol or bovine serum albumin (BSA) to the preincubation solution

Preincubation Incubated with:	Labelling Incubated with:	Intern. Fe (% Control)
MEM (control)	Tf	100±7
FAC	Tf	218±15
MEM + Ceruloplasmin (85 U/ml)	Tf	113±6
FAC + Ceruloplasmin (85 U/ml)	Tf	90±1
MEM + Ascorbate (0.5 mM)	Tf	117±7
FAC + Ascorbate (0.5 mM)	Tf	156±4
MEM + Ascorbate (5 mM)	Tf	95±5
FAC + Ascorbate (5 mM)	Tf	104±6
MEM + DFO (0.5 mM)	Tf	101±8
FAC + DFO (0.5 mM)	Tf	90±6
MEM + Hepes (20 mM)	Tf	105±5
FAC + Hepes (20 mM)	Tf	112±4
MEM + Mannitol (5 mM)	Tf	99±1
FAC + Mannitol (5 mM)	Tf	115±1
MEM + BSA (50 mg/ml)	Tf	115±4
FAC + BSA (50 mg/ml)	Tf	80±4

Cells were preincubated with MEM (control) or MEM containing FAC (25 μg/ml) in the presence of various free radical scavengers for three 45 min incubations at 37°C. This medium was removed and the cells then labelled with ^{59}Fe-^{125}I-Tf (0.1 mg/ml) for 2 h at 37°C. Results are mean ± S.D. (3 determinations) from a typical experiment of 2–3 experiments performed.

(50 mg/ml; [48]) when added with FAC almost completely prevented FAC-activated ^{59}Fe uptake (Table 6). Titration experiments demonstrated that BSA added to FAC at a concentration of 1 mg/ml had no effect on FAC-stimulated Fe uptake and that concentrations greater than 30 mg/ml were necessary to completely block this Fe uptake process. The ferroxidase, ceruloplasmin (Table 6), which prevents free radical formation by oxidising Fe(II) to Fe(III) [49], also prevented FAC-activated ^{59}Fe uptake. Furthermore, all these agents when added to MEM alone had no effect on ^{59}Fe uptake from Tf. Also, as found for Tf, the addition of catalase and HEPES to FAC markedly prevented the stimulated ^{59}Fe uptake seen from ^{59}Fe-citrate (Table 7). These data suggest that the initial activation event may be due to the production of free radicals from FAC via the Fe-catalysed Haber–Weiss reaction. For proper interpretation of these results, understanding of the redox chemistry involved is required, and is summarised in the equations below:

$$Fe(III) + O_2^{\cdot-} \rightleftharpoons Fe(II) + O_2 \quad (1)$$

$$Fe(II) + H_2O_2 \rightleftharpoons OH^{\cdot} + OH^- + Fe(III) \quad (2)$$

$$Net: O_2^{\cdot-} + H_2O_2 \overset{\text{iron catalyst}}{\rightleftharpoons} O_2 + OH^{\cdot} + OH^- \quad (3)$$

Eq. (2) is the Fenton reaction [50]; eq. (3) is the iron-catalysed Haber-Weiss reaction [51]. Hence, the active oxygen species involved in stimulating Fe uptake may be the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and or the hydroxyl radical ($OH^{\cdot}$) [50]. Superoxide dismutase removes $O_2^{\cdot-}$, whereas catalase removes H_2O_2, and both proteins effectively prevented FAC-activated Fe uptake (Table 5). It is of interest that both catalase and SOD can inhibit $OH^{\cdot}$ production by $O_2^{\cdot-}$-generating systems containing Fe [52]. Furthermore, Hepes, mannitol, DFO and high concentrations of BSA or ascorbic acid, have been demonstrated to be scavengers of $OH^{\cdot}$ [47,53], and all markedly prevented FAC-activated Fe uptake (Table 6).

To further investigate the role of free radicals in the activation event, cells were preincubated with the $O_2^{\cdot-}$-generating agent, methylene blue (MB; [54]). When cells were preincubated with MB (0.1 mM) alone, and then incubated with ^{59}Fe-^{125}I-Tf, there was a decrease in internalised Fe uptake to 52% of the control. In contrast, the addition of MB to FAC resulted in a marked increase in Fe uptake to 302% of the control (results not shown). Similarly, the addition of another $O_2^{\cdot-}$-generator, menadione (10 μM; [55]), to FAC also markedly stimulated Fe uptake from ^{59}Fe-citrate (Table 8), whereas preincubation with menadione alone was far less effective at increasing Fe uptake. When H_2O_2 (0.9 μM) was added to cells it had no effect on Fe uptake from Fe-citrate, whereas the addition of

Table 7
The effect of catalase and the buffer Hepes on internalised ^{59}Fe uptake from ^{59}Fe-citrate complexes by melanoma cells

Preincubation Incubated with:	Labelling Incubated with:	Internalised iron (μmole Fe/gPR)	Internalised Iron (%Control)
MEM (control)	Fe-citrate	0.04	100
FAC + MEM	Fe-citrate	0.82	2050
Catalase + MEM	Fe-citrate	0.04	100
Catalase + FAC	Fe-citrate	0.18	450
Hepes + MEM	Fe-citrate	0.04	100
Hepes + FAC	Fe-citrate	0.14	350

Melanoma cells were preincubated with MEM or MEM-containing FAC (25 μg/ml) and also MEM- or FAC-containing catalase (1000 U/ml) or Hepes (20 mM) for three 45 min incubations at 37°C. After this procedure, the cells were then labelled with ^{59}Fe-citrate (2.5 μM) for 2 h at 37°C. Results are means of 2 determinations in a typical experiment of 3 experiments performed. The variation between duplicates was less than 5%.

Table 8
The effect of the superoxide generator, menadione (Men), and also hydrogen peroxide (HP), on internalised ^{59}Fe uptake from ^{59}Fe-citrate complexes by melanoma cells

Preincubation	Labelling	Internalised iron	
Incubated with:	Incubated with:	(μmole Fe/gPR)	(% Control)
MEM (control)	Fe-citrate	0.03	100
FAC + MEM	Fe-citrate	0.64	2133
Men + MEM	Fe-citrate	0.07	233
Men + FAC	Fe-citrate	1.86	6200
HP + MEM	Fe-citrate	0.03	100
HP + FAC	Fe-citrate	0.89	2967

Cells were preincubated with MEM or MEM-containing FAC (25 μg/ml), or MEM- or FAC (25 μg/ml)-containing Men (10 μM) or HP (0.9 μM) for three 45 min incubations at 37°C. After this procedure, the cells were then labelled with ^{59}Fe-citrate (2.5 μM) for 2 h at 37°C. Results are the means of 2 determinations in a typical experiment of 3 experiments performed. The variation between duplicates was less than 5%.

H_2O_2 to FAC stimulated Fe uptake, although the extent of stimulation was not as great as that observed in the presence of menadione (Table 8).

The results described above suggest that neither H_2O_2 nor $O_2^{\cdot-}$ alone were responsible for activation of the FAC-stimulated mechanism. However, the addition of H_2O_2 and $O_2^{\cdot-}$ to FAC would result in an increase in the production of $OH^{\cdot}$ via the Fe-catalysed Haber–Weiss reaction (Eq. 3; [51]), and considering these data together with that listed in Table 5 and 6, it can be suggested that $OH^{\cdot}$ may be the active agent. When the $O_2^{\cdot-}$ generator, menadione, was added to FAC, it was far more effective than H_2O_2 at stimulating ^{59}Fe uptake (Table 8). Relevant to this, Kaplan et al. [17] have demonstrated that Fe in the FAC complex is mainly in the Fe(III) oxidation state, with very little present as Fe(II). Hence, under the present experimental conditions, the production of Fe(II) is probably the rate-limiting step in the Fe-catalysed Haber–Weiss reaction, since the addition of $O_2^{\cdot-}$ results in the reduction of Fe(III) to Fe(II) (see Eq. 1), which drives the reaction towards the production of $OH^{\cdot}$ (Eq. 3). Similarly, this may explain why SOD inhibits the FAC-activated Fe uptake process (Table 5), as the reduction of Fe(III) to Fe(II) would not occur in the absence of $O_2^{\cdot-}$.

4. Discussion

The present study suggests that the initial stimulation of FAC-activated Fe uptake from Tf and Fe-citrate may be caused by the production of $OH^{\cdot}$ via the Fe-catalysed Haber–Weiss reaction. This mechanism of Fe uptake is not only found in human melanoma cells, but it is also present in human fibroblasts, a human embryonic kidney cell line and Chinese hamster ovary cells, suggesting that it may be of widespread significance. In addition, Kaplan et al. [17] examining the stimulatory effect of FAC on Fe uptake from Fe-NTA, also found that this process is present in a wide range of cell types, including skin fibroblasts, HeLa cells, L-cells and a hepatocellular carcinoma (Hep-G2) cell line. However, these latter authors did not establish the mechanism of how FAC stimulated Fe uptake or the fact that preincubation of cells with FAC can also markedly increase Fe uptake from Tf. Our present experiments clearly demonstrate that FAC-activated Fe uptake from Tf is not due to either RME or the postulated diferric Tf reductase, but represents a previously unrecognised mechanism which may also be involved in Fe uptake from small M_r complexes. When FAC-activates this process, Fe is efficiently internalised within the cell (Fig. 2a and b) where it can be used for metabolic functioning or stored in ferritin. Moreover, Fe accumulated after treatment with FAC enters physiologically relevant intracellular Fe pools, as it results in an accumulation of ferritin-Fe and down-regulation of the TfR [17,25].

The observation that $OH^{\cdot}$ can markedly stimulate Fe uptake may have important physiological and pathophysiological consequences. There is a large body of evidence suggesting that free radicals play an important role in the pathogenesis of many human diseases, including Fe overload [47,56–61]. The $OH^{\cdot}$ radical can be formed under physiological and pathophysiological conditions in vivo by (A), the Fe-catalysed Haber–Weiss reaction, utilising $O_2^{\cdot-}$ generated by normal aerobic metabolism or via xanthine oxidase in post-ischaemic tissues [62], and (B) via the Fe-independent peroxynitrite pathway [63]. In Fe overload disease for example, high concentrations of serum non-Tf-bound Fe exists in the form of Fe-citrate [33,64], which is capable of creating free radicals via the Fe-catalysed Haber–Weiss reaction [65,66]. In these diseases non-Tf-bound Fe can reach concentrations higher than those used in our experiments (i.e., > 70 μM; [60,65]), and hence, the Fe-loading seen in these diseases may be partly due to the $OH^{\cdot}$-activated Fe uptake process. It can be suggested that by removing extracellular low-M_r Fe and storing it intracellularly in ferritin for example, the cell prevents $OH^{\cdot}$-mediated lipid peroxidation of its plasma membrane and the consequential cellular injury or death.

High concentrations of BSA prevented the non-specific interaction of Tf with the cell membrane that was necessary for FAC-activated Fe uptake. Moreover, ceruloplasmin and high concentrations of BSA also prevented stimulation when added to FAC (Table 6), probably due to their ferroxidase [49] and non-specific free radical scavenging properties [48], respectively. Therefore, in the plasma, ceruloplasmin and the high concentrations of serum proteins may prevent the FAC-activated process. However, an albumin concentration of > 30 mg/ml was essential to completely prevent activation when BSA was added to FAC, and a BSA concentration of 70 mg/ml was required to preclude the non-specific binding of Tf that was necessary for FAC-activated Fe uptake. These observations are

crucial regarding the physiological relevance of the FAC-stimulated mechanism, as the concentrations of albumin and ceruloplasmin are far reduced in the interstitial fluid that comes into direct contact with cells. For instance, while the protein concentration of plasma is ≈ 73 mg/ml ([albumin] = 44 mg/ml), the protein concentration of the interstitial fluid at the arterial ends of capillaries may be as low as 3 mg/ml ([albumin] = 1.8 mg/ml; [67]). Schmidt and Thews [67] have stated that an acceptable estimate of the mean protein concentration of the interstitial fluid is 18–20 mg/ml ([albumin] = 11–12 mg/ml), and under these conditions the FAC-activated mechanism could take place. Also, in Fe overload disease where there is a high concentration of non-Tf-bound Fe, there is evidence that antioxidant defenses are overwhelmed. For example, despite elevated ceruloplasmin concentrations in haemochromatosis patients with high levels of non-Tf-bound Fe, serum from these patients can stimulate lipid peroxidation [65]. This report suggests that in vivo non-Tf-bound Fe results in oxidative damage despite the presence of albumin and ceruloplasmin, and under these circumstances the FAC-activated process may occur.

It can be argued that the concentration of Tf in the interstitial fluid is also reduced compared to the plasma. However, in Fe overload disease Tf is almost fully saturated with Fe [2], and considering that the plasma Tf concentration is ≈ 2.3 mg/ml [2], and that the Tf concentration of the interstitial fluid is about one-third this value (0.8 mg/ml; [68]), it is clear that this Tf concentration is far greater than that required for saturation of the TfR (0.01 mg/ml; [19,25]). Therefore, the Tf concentration in the interstitial fluid is probably suitable for acting as a substrate for the FAC-activated Fe uptake process.

Considering the question of how $OH^{\cdot}$ activates this Fe uptake mechanism, it can be suggested that a genetic mechanism is probably not involved, since exposure of cells to FAC for only 5 min was sufficient to produce enhanced Fe uptake after a 2 h incubation with Tf. Kaplan et al. [17], investigating the effect of FAC on Fe uptake from Fe-NTA by fibroblasts, have suggested that the increased rate of Fe uptake may be due to the recruitment of a cryptic pool of transporters to the cell surface. Such a mechanism could occur quite rapidly and may explain the results of the present investigation. However, further studies are required to characterise the molecular mechanism responsible for FAC-activated Fe uptake.

In conclusion, incubation of cells with FAC results in the production of $OH^{\cdot}$ which activates a membrane-bound Fe transport system that is capable of binding and transporting Fe in the form of low-M_r complexes. Furthermore, as a secondary consequence of its activity, this $OH^{\cdot}$-stimulated Fe uptake system can also remove Fe from Tf when the Tf becomes non-specifically adsorbed to the cell membrane. This latter observation could indicate that processes of reduction and/or protonation are occurring which are sufficient to remove Fe from the high affinity binding sites of Tf. We speculate that the FAC-activated Fe uptake process represents a protective mechanism to guard against oxidant stress generated from low-M_r Fe complexes which may be important in pathophysiological states such as Fe-overload disease.

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References

[1] Kühn, L.C., Schulman, H.M. and Ponka, P. (1990) in Iron Transport and Storage (Ponka, P., Schulman, H.M. and Woodworth, R.C., eds.), pp. 149–193, CRC Press, Boca Raton, FL.
[2] Morgan, E.H. (1981) Mol. Aspects Med. 4, 1–123.
[3] Klausner, R.D., Ashwell, G., Van Renswoude, J., Harford, J.B. and Bridges, K.R. (1983) Proc. Natl. Acad. Sci. USA 80, 2263–2266.
[4] Nunez, M.-T., Gaete, V., Watkins, J.A. and Glass, J. (1990) J. Biol. Chem. 265, 6688–6692.
[5] Crane, F.L., Sun, I.L., Clarke, M.G., Grebing, C. and Low, H. (1985) Biochim. Biophys. Acta 811, 233–264.
[6] Crane, F.L., Sun, I.L., Barr, R. and Low, H. (1991) J. Bioenerg. Biomembr. 23(5), 773–804.
[7] Sun, I.L., Navas, P., Crane, F.L., Morre, D.J. and Low, H. (1987) J. Biol. Chem. 262, 15915–15921.
[8] Thorstensen, K. (1988) J. Biol. Chem. 263, 16837–16841.
[9] Thorstensen, K. and Romslo, I. (1988) J. Biol. Chem. 263, 8844–8850.
[10] Goldenberg, H., Dodel, B. and Seidl, D. (1990) Eur. J. Biochem. 192, 475–480.
[11] Thorstensen, K. and Aisen, P. (1990) Biochim. Biophys. Acta 1052, 29–35.
[12] Titeux, M., Testa, U., Louache, F., Thomopoulos, P., Rochant, H. and Breton-Gorius, J. (1984) J. Cell. Physiol. 121, 251–256.
[13] Wright, T.L., Brissot, P., Ma, W.-L. and Weisiger, R.A. (1986) J. Biol. Chem. 261, 10909–10914.
[14] Wright, T.L., Fits, J.G. and Weisiger, R.A. (1988) J. Biol. Chem. 263, 1842–1847.
[15] Sturrock, A., Alexander, J., Lamb, J., Craven, C.M. and Kaplan, J. (1990) J. Biol. Chem. 265, 3139–3145.
[16] Richardson, D.R. and Baker, E. (1991) Biochim. Biophys. Acta 1093, 20–28.
[17] Kaplan, J., Jordan, I. and Sturrock, A. (1991) J. Biol. Chem. 266, 2997–3004.
[18] Craven, C.M., Alexander, J., Eldridge, M., Kushner, J.P., Bernstein, S. and Kaplan, J. (1987) Proc. Natl. Acad. Sci. USA 84, 3457–3461.
[19] Richardson, D.R. and Baker, E. (1990) Biochim. Biophys. Acta 1053, 1–12.
[20] Richardson, D.R. and Baker, E. (1994) J. Cell. Physiol. 161, 160–168.
[21] Brown, J.P., Hewick, R.M., Hellström, I., Hellström, K.E., Doolittle, R.F. and Dreyer, W.J. (1982) Nature 296, 171–173.
[22] Richardson, D.R. (1990) The mechanisms of iron and transferrin uptake by the human malignant melanoma cell, Ph.D. thesis, University of Western Australia, Perth, Nedlands, 6009.
[23] Richardson, D.R. and Baker, E. (1991) Biochim. Biophys. Acta 1091, 294–302.

[24] Richardson, D.R. and Baker, E. (1992) Biochim. Biophys. Acta 1103, 275–280
[25] Richardson, D.R. and Baker, E. (1992) J. Biol. Chem. 267, 13972–13979.
[26] McGraw, T.E., Greenfield, L. and Maxfield, F.R. (1987) J. Cell. Biol. 105, 207–214.
[27] Goto, Y., Paterson, M. and Listowsky, I. (1983) J. Biol. Chem. 258, 5248–5255.
[28] Ward, J.H., Kushner, J.P. and Kaplan, J. (1982) J. Biol. Chem. 257, 10317–10323.
[29] Ward, J.H., Jordan, I., Kushner, J.P. and Kaplan, J. (1984) J. Biol. Chem. 259, 13235–13240.
[30] Spiro, T.G., Pape, L. and Saltman, P. (1967) J. Am. Chem. Soc. 89, 5555–5559.
[31] Spiro, T.G., Bates, G. and Saltman, P. (1967) J. Am. Chem. Soc. 89, 5559–5562.
[32] Inman, R.S. and Wessling-Resnick, M. (1993) J. Biol. Chem. 268, 8521–8528.
[33] Grootveld, M., Bell, J.D., Halliwell, B., Aruoma, O.I., Bomford, A. and Sadler, P.J. (1989) J. Biol. Chem. 264, 4417–4422.
[34] Karin, M. and Mintz, B. (1981) J. Biol. Chem. 256, 3245–3252.
[35] Richardson, D.R. and Baker, E. (1992) J. Biol. Chem. 267, 21384–21389.
[36] Low, H., Sun, I.L., Navas, P., Grebing, C., Crane, F.L. and Morre, D.J. (1986) Biochem. Biophys. Res. Commun. 139(3), 1117–1123.
[37] Low, H., Grebing, C., Lindgren, A., Tally, M., Sun, I.L. and Crane, F.L. (1987) J. Bioenerg. Biomembr. 19, 535–549.
[38] Sun, I.L., Crane, F.L., Low, H. and Grebing, C. (1984) Biochem. Biophys. Res. Commun. 125(2), 649–654.
[39] Sun, I.L., Toole-Simms, W., Crane, F.L., Morre, D.J., Low, H. and Chou, J.Y. (1988) Biochim. Biophys. Acta 938, 17–23.
[40] Allnutt, F.C.T. and Bonner, W.D. (1987) Plant Physiol. 85, 746–750.
[41] Morgan, E.H. (1981) Biochim. Biophys. Acta 642, 119–134.
[42] Trowbridge, I.S. and Lopez, F. (1982) Proc. Natl. Acad. Sci. USA 79, 1175–1179.
[43] Chan, R.Y.Y., Ponka, P. and Schulman, H.M. (1992) Exp. Cell. Res. 202, 326–336.
[44] McAbee, D.D. and Esbensen, K. (1991) J. Biol. Chem. 266, 23624–23631.
[45] Hradilek, A. and Neuwirt, J. (1986) Br. J. Haematol. 62, 21–30.
[46] Davies, K.J.A., Delsignore, M.E. and Lin, S.W. (1987) J. Biol. Chem. 262, 9902–9907.
[47] Halliwell, B. and Gutteridge, J.M.C. (1989) Free Radicals in Biology and Medicine, 2nd Ed., Clarendon Press, Oxford.
[48] Halliwell, B. (1988) Biochem. Pharmacol. 37, 569–571.
[49] Ryan, T.P. and Aust, S.D. (1992) Crit. Rev. Toxicol. 22(1), 119–141.
[50] Fenton, H.J.H. (1894) J. Chem. Soc. 65, 899–910.
[51] Haber, F. and Weiss, J. (1934) Proc. R. Soc. Edinburgh Sect. A 147, 332–351.
[52] Rowley, D.A. and Halliwell, B. (1982) Febs Lett. 138(1), 33–36.
[53] Grady, J.K., Chasteen, N.D. and Harris, D.C. (1988) Anal. Biochem. 173, 111–115.
[54] McCord, J.M. and Fridovich, I. (1970) J. Biol. Chem. 245, 1374–1377.
[55] Thor, H., Smith, M.T., Hartzell, P., Bellomo, G., Jewell, S.A. and Orrenius, S. (1982) J. Biol. Chem. 257, 12419–12425.
[56] Wills, E.D. (1972) Biochem. Pharmacol. 21, 239–247.
[57] Rachmilewitz, E.A., Lubin, B.H. and Shohet, S.B. (1976) Blood 47, 495–505.
[58] Heys, A.D. and Dormandy, T.L. (1981) Clin. Sci. 60, 295–301.
[59] Freeman, B.A. and Crapo, J.D. (1982) Lab. Invest. 47, 412–426.
[60] Hershko, C. and Peto, T.E.A. (1987) Br. J. Haematol. 66, 149–151.
[61] Winyard, P.G., Blake, D.R., Chirico, S., Gutteridge, J.M.C., Lunec, J. (1987) Lancet i, 69.
[62] Chance, B., Sies, H. and Boveris, A. (1979) Physiol. Rev. 59, 527–605.
[63] Schmidt, H.H.H.W. (1992) Febs Lett. 307(1), 102–107.
[64] Hershko, C., Graham, G., Bates, G.W. and Rachmilewitz, E.A. (1978) Brit. J. Haematol. 40, 255–263.
[65] Gutteridge, J.M.C., Rowley, D.A., Griffiths, E. and Halliwell, B. (1985) Clin. Sci. 68, 463–467.
[66] Halliwell, B. and Gutteridge, J.M.C. (1986) Arch. Biochem. Biophys. 246, 501–514.
[67] Schmidt, R.F. and Thews, G. (1983) Human Physiology, p. 419, Springer, Berlin.
[68] Barber, B.J., Schultz, T.J. and Randlett, D.L. (1990) Am. J. Physiol. 258, G714–718.