Lipoic Acid Protects Efficiently Only against a Specific Form of Peroxynitrite-induced Damage*

Bashir M. Rezkt, Guido R. M. M. Haenen‡, Wim J. F. van der Vijgh‡,§, and Aalt Bast‡

From the ‡Department of Pharmacology and Toxicology, Faculty of Medicine, Universiteit Maastricht; P.O. Box 616, NL 6200 MD Maastricht, The Netherlands and the §Department of Medical Oncology, Vrije Universiteit Medical Center; De Boelelaan 1117, NL 1081 HV Amsterdam, The Netherlands

The ability of the sulfur-containing compounds glutathione (GSH), glutathione disulphide (GSSG), S-methylglutathione (GSMe), lipoic acid (LA), and dihydrolipoic acid (DHLA) to protect against hypochlorous acid (HOCl)-mediated damage and peroxynitrite (ONOOH)-induced damage has been compared. Protective activity was assessed in competition assays by monitoring several detectors, i.e. dihydrorhodamine-123 (DHR-123) oxidation, α1-antiproteinase (α1-AP) inactivation, and glutathione S-transferase P1-1 (GST-P1-1) inactivation. In addition, nitration of tyrosine was measured to assess protection of the sulfur-containing compounds against ONOOH. For protection against HOCl, the efficacy of the antioxidant was controlled by the ratio of the reaction rates of the antioxidant and the detector molecule with the oxidant. The rank order of the activity of the antioxidants (GSH > DHLA > LA > GSMe > GSSG) appeared to be independent of the detector used. However, the rank order of the antioxidants against ONOOH-induced damage is strongly dependent on the detector. LA was 40 times less active than GSH in the inhibition of ONOOH-induced DHR-123 oxidation, whereas LA was 20 times more active than GSH in preventing the inhibition of GST-P1-1 by ONOOH. This points to different molecular mechanisms of ONOOH damage to DHR-123 compared with ONOOH damage to GST-P1-1. LA is a poor antioxidant in protecting against the form of ONOOH damage involved in DHR-123 oxidation. In the form of ONOOH toxicity involved in GST-P1-1 inhibition, LA is the most potent sulfur-containing antioxidant in our series. It is proposed that an intermediate product in which both sulfur atoms of LA have reacted is involved in the reaction of ONOOH with LA. The high potency of LA to protect GST-P1-1 against ONOOH might be of therapeutic interest.

Some sulfur-containing compounds are efficient antioxidants and can protect against damage induced by peroxynitrite (ONOOH) (1–5) and hypochlorous acid (HOCl) (6, 7). ONOOH and HOCl are highly effective antibacterial agents formed by neutrophils in innate immunity (8). Uncontrolled and excessive production of the reactive species, however, will cause collateral damage to surrounding tissue at the site of inflammation, e.g. low density lipoprotein (LDL) oxidation or DNA, RNA, and protein damage (9–13). ONOOH is known to mediate both one- and two-electron oxidation reactions, whereas HOCl predominantly gives two-electron oxidation (14, 15).

It has been found that lipoic acid (LA) is a very potent protector against ONOOH-mediated damage (1–4). In contrast, preliminary studies in our laboratory indicated that LA is a very poor protector against ONOOH-mediated oxidation of dihydrorhodamine-123 (DHR-123). This controversy might originate from differences in the procedures used to assess the antioxidant activity of LA. The activity of an antioxidant is usually determined by quantifying the ability of the antioxidant to prevent damage induced by a reactive species to a detector. Den Hartog et al. (7) emphasized that the efficacy of an antioxidant depends on the reaction rate of the oxidant with the detector molecule relative to the reaction rate of the oxidant with the antioxidant. This was illustrated by the difference in protective activity against the HOCl of several sulfur-containing antioxidants using different detectors (7). The slower the reaction of the detector with the oxidant, the higher was the apparent activity of a protector. The objective of the present study is to further examine the antioxidant activity of sulfur-containing compounds in the presence of different detectors (protein and non-protein) and different oxidants (ONOOH and HOCl) in order to resolve the apparent discrepancy in the efficacy of LA in several antioxidant assays. The results show that, with HOCl, the rate of the reaction of HOCl with the oxidant relative to that of the detector is essential. With ONOOH, the molecular mechanism of damage and protection is decisive for the efficacy of the antioxidant.

MATERIALS AND METHODS

Chemicals—Elastase, α1-antiproteinase (α1-AP), glutathione S-transferase P-1-1 (GST-P1-1), GSH, GSSG, GSMe, 1-chloro-2,4-dinitrobenzene (CDNB) l-tyrosine, 3-nitro-l-tyrosine, 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), and sodium hypochlorite (NaOCl) were obtained from Sigma. DHR-123 and KOr2 were obtained from Fluca Chemie GmbH (Buchs, Switzerland). LA and β-LA were obtained from Asta Medica AG (Frankfurt, Germany). Nitrogen monoxide was obtained from AGA (Hamburg, Germany). All other chemicals were of the highest grade of purity.

Synthesis of Potassium Oxoperonitrate—Potassium oxoperonitrate was produced from the reaction of solid KOr2 with NO gas as described by Koppennol et al. (16). Briefly, the NO gas was slowly led over the mixture of KOr2 and quartz sand, which was constantly stirred and kept on ice. The mixture was poured into a cold potassium hydroxide solution. Manganese dioxide was used to remove the hydrogen peroxide that results from decomposition of the excess of potassium superoxide. A solution with ONOOH was obtained by filtering off the sand and manganese dioxide. The concentration of ONOOH was determined spectrophotometrically at 302 nm.

Oxidation of DHR-123—The use of DHR-123 as a detector to monitor
Selective Protection against Peroxynitrite by Lipoic Acid

Protection against ONOO- and HOCl-induced oxidation was based on the procedure described by Kooy et al. (17). In short, 100 μl of the desired concentration of the test compound was added to 0.9 ml of 10 mM phosphate buffer at 37 °C. DHR-123 was added to give a final concentration of 5 μM. During vortexing, 10 μl of ONOOH or HOCl was added to give a final concentration of 0.6 or 5 μM, respectively. The fluorescent product, rhodamine-123, was measured by fluorescence detection with excitation and emission wavelengths of 500 and 536 nm, respectively. The effects are expressed as the concentration giving 50% protection with excitation and emission wavelengths of 500 and 536 nm, respectively. Control experiments showed that the test compounds in a concentration of 500 μM did not affect the fluorescence.

Elastase Assay—Elastase activity was used as a detector according to the procedure described by Haenen and Bast (6) with minor modifications. The reagents were dissolved in potassium phosphate buffer (19 mM), pH 7.4, containing 140 mM NaCl. Twenty micrograms of α1-AP was preincubated at 37 °C for 5 min with the test compounds in the desired concentration. During vortexing, 10 μl of ONOOH was added. The final concentration of ONOOH was 150 μM. After 5 min incubation, 5 μg of elastase was added. After 10 min of incubation, 50 μl of a 10 mM solution of N-tetra-butylcarbonyl-1-alanine p-nitrophenyl ester in methanol was added. The increase in absorption at 410 nm was determined. Control experiments showed that the test compounds in a concentration of 100 μM did not affect the activity of control or ONOOH-inactivated α1-AP.

Glutathione-S-transferase P1-1 Assay—GST-P1-1 (5 μg/ml) was mixed with the test compound in 100 mM potassium phosphate buffer (pH 6.5), and the mixture was incubated at 37 °C for 1 min. During vortexing, ONOOH (10 μM, final concentration) was added. After 2 min of incubation, 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH were added. GST-P1-1 activity was monitored spectrophotometrically by recording the increase in absorbance at 340 nm (18).

Nitrotyrosine Assay—Under vortexing, 10 μl of ONOOH solution (final concentration 150 μM) was added to a solution containing 100 μM l-tyrosine in 100 mM potassium phosphate buffer (pH 7.4) and the test compound in the desired concentration. This mixture was incubated for 5 min. Measurement of 3-nitrotyrosine was carried out by using HPLC.

The results of these sulfur-containing compounds against HOCl-mediated inhibition. LA, a compound that contains an intramolecular disulfide, was hardly effective in the prevention of DHR-123 oxidation by ONOOH, but it potently protected against ONOOH-induced α1-AP damage and tyrosine nitration. Remarkably, both LA and DHLA had a very potent protective activity against ONOOH when GST-P1-1 was used as a detector. Their protective effect against ONOOH with this detector was ~20 times more potent than that of GSH.

The protective effect of these sulfur-containing compounds against HOCl between GSH and LA prompted us to perform additional experiments. The consumption of GSH and LA by different concentrations of ONOOH was determined (Fig. 1). The stoichiometry of the initial reaction of GSH was −1, i.e. one molecule of GSH reacts with one molecule of ONOOH. The apparent stoichiometry of LA was >1. With a 5-fold excess of ONOOH over LA, less than half of the LA was consumed. The product formed was β-LA, the thiosulfinate of LA that has four possible isomers (4, 6).

The stoichiometry of the initial reaction of GSH with HOCl was comparable with that of LA, i.e. one molecule of the antioxidant reacts with one molecule of the oxidant (Fig. 2). The product formed by the reaction of LA with HOCl was β-LA (Fig. 2).

RESULTS

Protection against HOCl was assessed in competition assays using the detector molecules DHR-123, α1-AP, and GST-P1-1 (Table I). Each of the sulfur-containing compounds tested had a prominent protective effect against HOCl except GSSG, which only had a moderate protective effect against HOCl with DHR-123 as a detector molecule. The protective effect of these sulfur-containing compounds against ONOOH was also assessed in competition assays using the detector molecules DHR-123, α1-AP, GST-P1-1, and tyrosine (Table II). GSH efficiently protected these detector molecules against ONOOH; the IC50 of GSH was practically independent of the detector. GSSG could not protect any of the detector molecules used against ONOOH. Methylation of GSH (giving GSSMe) abolished the ability to protect against oxidation of DHR-123 and nitration of tyrosine with ONOOH. GSSMe still had a considerable protective activity against ONOOH when α1-AP was used as a detector and, to a lesser extent, when GST P1-1 was used as a detector. Dihydrolipoic acid (DHLA), a dithiol, had a protective effect against ONOOH that was slightly lower than that of GSH when DHR-123 was used as a detector. DHLA efficiently protected α1-AP or tyrosine against ONOOH-mediated inhibition. LA, a compound that contains an intramolecular disulfide, was hardly effective in the prevention of DHR-123 oxidation by ONOOH, but it potently protected against ONOOH-induced α1-AP damage and tyrosine nitration. Remarkably, both LA and DHLA had a very potent protective activity against ONOOH when GST-P1-1 was used as a detector. Their protective effect against ONOOH with this detector was ~20 times more potent than that of GSH.

The difference in protective activity against ONOOH between GSH and LA prompted us to perform additional experiments. The consumption of GSH and LA by different concentrations of ONOOH was determined (Fig. 1). The stoichiometry of the initial reaction of GSH was −1, i.e. one molecule of GSH reacts with one molecule of ONOOH. The apparent stoichiometry of LA was >1. With a 5-fold excess of ONOOH over LA, less than half of the LA was consumed. The product formed was β-LA, the thiosulfinate of LA that has four possible isomers (4, 6).

The stoichiometry of the initial reaction of GSH with HOCl was comparable with that of LA, i.e. one molecule of the antioxidant reacts with one molecule of the oxidant (Fig. 2). The product formed by the reaction of LA with HOCl was β-LA (Fig. 2).

DISCUSSION

Some sulfur-containing compounds can form an effective line of defense against reactive oxygen and nitrogen species. The aim of the present study is to further examine the antioxidant activity of sulfur-containing compounds in order to explain the difference in activity observed in some assays. The present study demonstrated differences in the protective activity of sulfur-containing compounds against HOCl-mediated oxidation of different detectors. This can be explained by the rate of the reaction of the sulfur antioxidant with HOCl relative to the rate of the reaction of the detector with HOCl. The rank order of the antioxidants is independent of the detector used. The onsets activity of a poor antioxidant can be upgraded by using a slowly reacting detector. This is shown nicely by the relatively high potency of GSSG, a compound that reacts sluggishly with HOCl (6) to protect DHR-123 against HOCl-mediated oxidation (Table I), because DHR-123 reacts just as sluggishly with GSSG as GSSG does. GSSG does not protect faster reacting detectors, e.g. α1-AP and GST-P1-1, against HOCl damage. Similarly, Den Hartog et al. (7) reported that acetylcholinesterase is efficiently protected by GSSG against HOCl-induced damage. This can also be explained by the relatively

### Table I

| Compound             | IC50 DHR-123 | IC50 α1-AP | IC50 GST-P1-1 |
|----------------------|--------------|------------|---------------|
| Reduced glutathione  | 1.3 ± 0.5    | 20         | 0.3           |
| S-Methylglutathione  | 1.5 ± 0.7    | 35         | ND            |
| Glutathione disulfide| 9 ± 1        | >200       | >200          |
| Dihydrolipoic acid   | 5 ± 3        | 30         | 1.2           |
| Dihydrolipoic acid   | 3.3 ± 0.6    | 29         | ND            |

* Data taken from Haenen and Bast (6).
* Data taken from den Hartog et al. (7).

The protective activity of several sulfur-containing compounds against HOCl-induced damage assessed using different detectors

Detectors used are DHR-123 oxidation, α1-AP inactivation, and GST-P1-1 inhibition. The activity is expressed as the IC50 value, the concentration needed to reduce the HOCl-induced inhibition or oxidation by 50%. The lower the IC50, the more potent the compound. Values are presented as mean ± S.D. of at least three separate experiments. ND, no data.
The protecting activity of several sulfur-containing compounds against ONOOH-mediated damage assessed with different detectors

Detectors used are DHR-123 oxidation, α₁-AP inactivation, GST-P1-1 inactivation, and tyrosine nitration. The activity is expressed as the IC₅₀ value, the concentration needed to reduce the ONOOH-induced oxidation, inhibition, or nitration by 50%. The lower the IC₅₀, the more potent the compound. Values are presented as mean ± S.D. of at least three separate experiments.

| Compound                | IC₅₀ ONOOH-DHR-123 μM | IC₅₀ ONOOH-α₁-AP μM | IC₅₀ ONOOH-GST-P1-1 μM | IC₅₀ ONOOH-tyrosine μM |
|-------------------------|-----------------------|---------------------|------------------------|------------------------|
| Reduced glutathione     | 26 ± 9                | 38 ± 19             | 17 ± 1                 | 20 ± 1                 |
| S-Methylglutathione     | >1000                 | 52 ± 6              | 104 ± 3                | >200                   |
| Glutathione disulfide   | >1000                 | >500                | >200                   | >200                   |
| Lipoic acid             | 924 ± 66              | 17 ± 2              | 0.9 ± 0.1              | 24 ± 1                 |
| Dihydrolipoic acid      | 56 ± 9                | 17 ± 1              | 1.0 ± 0.1              | 23 ± 1                 |

**Fig. 1.** LA or GSH concentration after the addition of a varying initial concentration of ONOOH (0–500 μM) to a solution containing 100 μM LA or 100 μM GSH. The formation of β-LA from LA was also determined. The symbols are as follows: closed circle, GSH; closed triangle, LA; and open triangle, β-LA. Based on the GSH consumption at a low concentration of ONOOH, the stoichiometry of the reaction is ~1:1. Values are presented as mean ± S.D. of at least three separate experiments.

**Fig. 2.** LA or GSH concentration after addition of a varying initial concentration of HOCl (0–200 μM) to a solution containing 100 μM LA or 100 μM GSH. The formation of β-LA from LA was also determined. The symbols are as follows: closed circle, GSH; closed triangle, LA; and open triangle, β-LA. Based on the GSH or LA consumption, the stoichiometry of the reaction of GSH or LA with HOCl is 1:1. Values are presented as mean ± S.D. of at least three separate experiments.

The explanations of Whiteman et al. (2) for the very low IC₅₀ of LA compared with that of the initial concentration of ONOOH were, first, that one molecule of LA can scavenge several ONOOH molecules and, second, that LA can combine with reactive intermediates. The first explanation, *viz.* that LA...
Selective Protection against Peroxynitrite by Lipoic Acid

The oxidation products of LA that is formed by ONOOH is identical to that formed by HOCl (Figs. 1 and 2), i.e. β-LA. This two-electron oxidation product of LA is formed after a reaction of one LA with probably one ONOOH (4) or one HOCl (23). This makes it unlikely that LA reacts with several ONOOH molecules. The second explanation of Whiteman et al. (2), i.e. that LA can combine with the reactive intermediates of ONOOH, seems more plausible. Whiteman et al. (2) stated that these intermediate products of ONOOH could be NO$_2^-$ and NO$_2^+$.

Interestingly, Nakagawa et al. (3) reported reaction of ONOOH by LA with a stoichiometry of 1:1. This two-electron oxidation product of LA is formed after a reaction of one LA with probably one ONOOH (4) or one HOCl (23). This makes it unlikely that LA reacts with several ONOOH molecules. The second explanation of Whiteman et al. (2), i.e. that LA can combine with the reactive intermediates of ONOOH, seems more plausible. Whiteman et al. (2) stated that these intermediate products of ONOOH could be NO$_2^-$ and NO$_2^+$.

The major finding that is in conflict with this theory is the reported reaction of ONOOH by LA with a stoichiometry of 1:1 and a second order rate constant of 1400 m$^{-1}$ s$^{-1}$, which is almost identical to that of GSH (1350 m$^{-1}$ s$^{-1}$) (4). Trujillo and Radi (4) determined this rate constant by monitoring ONOOH consumption in a stopped-flow spectrophotometer. In the same study, the relatively low consumption of LA by ONOOH, identical to the observation depicted in Fig. 1, was also reported. This finding, i.e. the low consumption of LA in this study and study of Trujillo and Radi (4), is in conflict with the very high second order rate constant reported. The consumption of LA by increasing amounts of ONOOH should have been identical to that of GSH, as shown in Fig. 2 for HOCl. As shown in Fig. 1, this is clearly not the case for LA consumption by ONOOH. The low consumption of LA by ONOOH is more in line with the hypothesis that only a part of the ONOOH, i.e. the bipolar form, reacts with LA. It should be noted that Trujillo and Radi (4) calculated the apparent second order rate constant by dividing the observed rate of ONOOH consumption by the concentration of LA and the concentration of ONOOH. If only part of the ONOOH is able to react with LA, i.e. the bipolar form, the concentration of the bipolar form should have been used in the calculation instead of the total amount of ONOOH. Consequently, the apparent second order rate constant of the reaction of the bipolar form of ONOOH with LA would have been higher than the reported value of the rate of the second order reaction of ONOOH with LA. GSH reacts with both forms of ONOOH, and, therefore, the second order rate constant of GSH cannot be adjusted in the same way as for LA. This would make the true second order rate constant of the reaction of the bipolar form of ONOOH with LA higher than that with GSH. This could explain the higher efficacy of LA compared with GSH in the protection of GST-P1-1.

In conclusion, the results obtained from this study underline that, in the efficacy of a free radical scavenger, the reaction rate of the oxidant with the antioxidant relative to the reaction rate of the oxidant with the detector is a major determinant factor. This is illustrated by the difference in protection of the sulfur-containing compounds against HOCl-induced damage to different targets. The difference in protection against ONOOH of these sulfur-containing antioxidants obtained with different targets illustrated that the molecular mechanisms of the damage inflicted by the reactive species and the molecular mechanism of protection by the antioxidant is of equal importance as the rate for the efficacy of the free radical scavenger. ONOOH damage proceeds via at least two mechanisms. LA does not protect against all forms of ONOOH damage. LA is a poor scavenger of ONOOH in this study and at least half of the ONOOH is not consumed by LA. This is in line with the findings of Nakagawa et al. (3). The oxidation products of LA that is formed by ONOOH are identical to that formed by HOCl (Figs. 1 and 2), i.e. β-LA. This two-electron oxidation product of LA is formed after a reaction of one LA with probably one ONOOH (4) or one HOCl (23). This makes it unlikely that LA reacts with several ONOOH molecules. The second explanation of Whiteman et al. (2), i.e. that LA can combine with the reactive intermediates of ONOOH, seems more plausible. Whiteman et al. (2) stated that these intermediate products of ONOOH could be NO$_2^-$ and NO$_2^+$.
antioxidant in the form of ONOOH toxicity involved in DHR-123 oxidation. However, in the form of ONOOH toxicity involved in GST-P1-1 inactivation, LA is the most potent sulfur-containing antioxidant in our series. It is proposed that an intermediate product in which both sulfur atoms of LA have reacted is involved in the reaction of ONOOH with LA. The high potency of LA to protect GST-P1-1 against ONOOH might be of interest for the therapeutic action of this drug.

REFERENCES
1. Whiteman, M., and Halliwell, B. (1997) FEBS Lett. 414, 497–500
2. Whiteman, M., Tritschler, H., and Halliwell, B. (1996) FEBS Lett. 379, 74–76
3. Nakagawa, H., Sumiki, E., Takusagawa, M., Ito, N., Matsuhashi, Y., and Ozawa, T. (2000) Chem. Pharm. Bull. (Tokyo) 48, 261–265
4. Trujillo, M., and Raddi, R. (2002) Arch. Biochem. Biophys. 397, 91–98
5. Kirsch, M., Lehnig, M., Korth, H. G., Sustmann, R., and de Groot, H. (2001) Chemistry 7, 3313–3320
6. Haenen, G. R. M. M., and Bast, A. (1991) Biochem. Pharmacol. 42, 2244–2246
7. den Hartog, G. J. M., Haenen, G. R. M. M., Vegt, E., van der Vlijgh, W. J. P., and Bast, A. (2002) Biol. Chem. 383, 709–713
8. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998) Blood 92, 3007–3017
9. White, C. R., Brock, T. A., Chang, L. Y., Crapo, J., Briscoe, P., Ku, D., Bradley, W. A., Gianturco, S. H., Gore, J., Freeman, B. A., and Tarpey, M. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1044–1048
10. Jenner, A. M., Ruiz, J. E., Dunster, C., Halliwell, B., Mann, G. E., and Siow, R. C. (2002) Arterioscler. Thromb. Vasc. Biol. 22, 574–580
11. Raddi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A., (1991) J. Biol. Chem. 266, 4244–4250
12. den Hartog, G. J. M., Vegt, E., van der Vlijgh, W. J. P., Haenen, G. R. M. M., and Bast, A. (2002) Toxicol. Appl. Pharmacol. 181, 228–233
13. van Haagen, B. M. I. M., den Hartog, G. J. M., Evelo, C. T. A., Haenen, G. R. M. M., and Bast, A. (2001) Chem. Biol. Interact 138, 77–83
14. Tereizy, A. C., Thomas, S. R., Burr, J. A., Liedler, D. C., and Stocker, R. (2002) Circ. Res. 90, 333–339
15. Priyadarshini, K. I., Kapoor, S., and Naik, D. B. (2001) Chem. Res. Toxicol. 14, 567–571
16. Koppenol, W. H., Kissner, R., and Beckman, J. S. (1996) Methods Enzymol. 269, 286–302
17. Kooy, N. W., Royall, J. A., Ischiropoulos, H., and Beckman, J. S. (1994) Free Radic. Biol. Med. 16, 149–156
18. Mannervik, B., and Gatenberg, C. (1981) Methods Enzymol. 77, 331–332
19. Anderson, M. E. (1985) Methods Enzymol. 113, 548–555
20. Chen, H. J., Wu, S. B., and Chang, C. M. (2005) Arch. Biochem. Biophys. 415, 109–116
21. Evans, M. D., and Pryor, W. A. (1994) Am. J. Physiol. 266, L393-L411
22. Wong, P. S., Eisnerich, J. P., Reddy, S., Lopez, C. L., Cross, C. E., and van der Vliet, A. (2001) Arch. Biochem. Biophys. 394, 216–228
23. Biewenga, G. P., de Jong, J., and Bast, A. (1994) Arch Biochem. Biophys. 312, 114–120

Selective Protection against Peroxynitrite by Lipoic Acid

9697
Lipoic Acid Protects Efficiently Only against a Specific Form of Peroxynitrite-induced Damage
Bashir M. Rezk, Guido R. M. M. Haenen, Wim J. F. van der Vijgh and Aalt Bast

J. Biol. Chem. 2004, 279:9693-9697. doi: 10.1074/jbc.M312289200 originally published online December 29, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M312289200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 22 references, 5 of which can be accessed free at http://www.jbc.org/content/279/11/9693.full.html#ref-list-1