Oxidative Damage to Specific Proteins in Replicative and Chronological-aged Saccharomyces cerevisiae

COMMON TARGETS AND PREVENTION BY CALORIE RESTRICTION*© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

This paper is available online at http://www.jbc.org

Revised for publication, April 30, 2004

Gemma Reverter-Branchat, Elisa Cabiscol, Jordi Tamarit, and Joaquim Ros‡

From the Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina, Universitat de Lleida, 25008 Lleida, Spain

Received for publication, April 30, 2004

Published, JBC Papers in Press, May 27, 2004, DOI 10.1074/jbc.M404849200

Oxidative modifications of cellular components have been described as one of the main contributions to aged phenotype. In Saccharomyces cerevisiae, two distinct life spans can be considered, replicative and chronological. The relationship between both aging models is still not clear despite suggestions that these phenomena may be related. In this work, we show that replicative and chronological-aged yeast cells are affected by an oxidative stress situation demonstrated by increased protein carbonylation when compared with young cells. The data on the identification of these oxidatively modified proteins gives clues to better understand cellular dysfunction that occurs during aging. Strikingly, although in both aging models metabolic differences are important, major targets are almost the same. Common targets include stress resistance proteins (Hsp60 and Hsp70) and enzymes involved in glucose metabolism such as enolase, glyceraldehyde-3-P dehydrogenase, fructose-1,6-biphosphate aldolase, pyruvate decarboxylase, and alcohol dehydrogenase. In both aging models, calorie restriction results in decreased damage to these proteins. In addition, chronological-aged cells grown under glucose restriction displayed lowered levels of lipid peroxidation product lipofuscin. Intracellular iron concentration is kept almost unchanged, whereas in non-restricted cells, the values increase up 4–5 times. The pro-oxidant effects of such increased iron concentration would account for the damage observed. Also, calorie-restricted cells show undamaged catalase, which clearly appears carbonylated in cells grown at a high glucose concentration. These results may explain lengthening of the viability of chronological-aged cells and could have an important role in replicative life span extension by calorie restriction.

Reactive oxygen species (ROS)1 generated by aerobic metabolism cause oxidative damage to cell components such as proteins, DNA, and lipids. Such damage will lead to altered structure and loss of biological function (1, 2). Superoxide anions cause the release of iron-sulfur clusters of several enzymes leading to its inactivation (3). The hydroxyl radical generated by H2O2 and transition metals, such as iron or copper, or by radiolysis is involved in protein backbone fragmentation or modification of amino acid side chains (1). There are increasing evidence that accumulation of these dysfunctional molecules in the cell over a lifetime contributes to the aging phenotype (4, 5). In this context, the pioneering studies of Stadtman and co-workers (6, 7) describe that oxidatively modified proteins (measured as protein carbonylation) accumulate as a function of cell age in human erythrocytes and fibroblasts. Moreover, protein damage in premature aging diseases such as progeria and Werner’s syndrome was much higher than in age-matched normal individuals (7). Oxidative modification of proteins has also been associated to aging-related pathologies such as Alzheimer’s disease (8). Using a proteomic approach, the identification of specific damaged proteins provided new insights into mechanisms of this neurodegenerative disease (9–11).

In yeast cells, two types of aging have been described, chronological and replicative. Chronological aging refers to the ability of stationary cultures to maintain viability over time. Investigations on this field have been used as a valuable model to study oxidative damage and aging of post-mitotic tissues of higher organisms (2, 13, 14). The damaged cell components cannot be diluted in these non-dividing cells. Consistently, the systems that get rid of these damaged components or prevent such damage will contribute to an increased chronological life span. Stationary phase cells become more resistant to heat and oxidative stresses, and overexpression of Sod1 and Sod2 extends life span by 30%. The metabolism of these cells tends to accumulate glycerogen and trehalose, and the cell wall thickens (15). Respiration is the main source of energy obtained from previously stored nutrients. Cell division in budding yeast cells occurs a finite number of times. As cells grow old, they accumulate bud scars, increase their size, and become unfertile and the nucleolus fragments. At the metabolic level, glucose metabolism shifts from glycolysis toward gluconeogenesis, which depends on the function of snf1, a kinase involved in cellular-adaptive responses to glucose deprivation (reviewed in Ref. 16). The regulation of this replicative aging points toward the Sir2 protein, a class III histone deacetylase that consumes NAD+ and suppresses rDNA recombination (17). Such recombination leads to the formation of extrachromosomal rDNA circles, which accumulate in old cells and are involved in cell death (18).

Calorie restriction is one of the models that has been proven to extend life span from yeast to mammals (19, 20). Lengthening of yeast life span was described by limiting the available

* This work was supported by grants BMC2001-0874 from Ministerio de Ciencia y Tecnología (Spain) and SGR 00128 from Generalitat de Catalunya. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. Ciències Mèdiques Bàsiques, Facultat de Medicina, Montserrat Roig, 2, 25008 Lleida, Spain. Tel.: 34-973-702-275; Fax: 34-973-702-426; E-mail: joaquim.ros@cmb.udl.es.

‡ The abbreviations used are: ROS, reactive oxygen species; Hsp, heat shock protein; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid.
glucose (0.5%) in a sir2-dependent manner or by reducing the activity of glucose-sensing cyclic AMP-dependent kinase (21, 22). Cultures with limited glucose showed increased respiration compared with 2% glucose. Interestingly, overexpression of transcription factor Hap4, which triggers the expression of genes involved in aerobic respiration, also extended life span even in 2% glucose (22). Because under aerobic respiration the NAD+/NADH ratio differs with respect to fermenting conditions, changes in this ratio would regulate Sir2 activity under calorie restriction (22, 23).

Aging studies in budding yeast have reported recently that (i) carbonylated proteins accumulate with replicative age and (ii) oxidized proteins are not inherited by daughter cells (24). In this work, we have identified oxidatively damaged proteins produced as the yeast cell age as well as other markers such as lipofuscin accumulation, iron concentration, and lipid peroxidation. The effect of calorie restriction on these parameters has also been analyzed. A survey of enzyme activities of the main protein targets indicated that oxidative modification of catalase can play an important role on the cell viability in chronological aging.

EXPERIMENTAL PROCEDURES

Organisms and Culture Conditions—The strain used throughout this work was Saccharomyces cerevisiae CML128 (25). Yeast cells were grown in rich YPD medium (1% yeast extract, 2% peptone, 2% glucose) or under calorie restriction (YPD medium with 0.5% glucose) by incubation in a rotary shaker at 30 °C. Cell viability was compared by spreading serial dilutions of the cultures in YPD plates with solid medium and incubation at 30 °C for 3 days. When indicated, cell growth was performed in synthetic medium either with 2 or 0.5% glucose and supplemented with amino acids, adenine and uracil, as described previously (26).

Isolation of Replicative Old Mother Cells—Old cells (16–18-generation-old) were purified by a modification of the biotin-streptavidin magnetic sorting procedure (27). 60 ml of exponentially growing cells to an OD 600 of 0.5 was centrifuged, resuspended in the respective medium, and sorted in a magnet. Unbound cells were removed, and the sorting procedure was repeated twice, resulting in 16–18-generation-old cells. In the last sorting separation, the purification step was repeated three times. At the end, cells were collected by centrifugation and stored at −20 °C. In our hands and compared with published procedures, (i) more cells could be isolated in each experiment, (ii) final recovery was better, and (iii) the amount of medium needed was reduced. To determine age and purity, bud scars were counted after labeling with fluorescent brightener (Calcofluor) followed by fluorescent microscopy visualization as described previously (27).

Preparation of Cell Extracts—Cells isolated by the sorting procedure were resuspended in 100 mM Tris-HCl buffer, pH 7.5, plus 10 mM dithiothreitol and treated with zymolase at 30 °C for 90 min to digest the cell wall. A concentrated solution of SDS previously heated at 95 °C was added (final SDS concentration: 6%) and incubated for 5 min at 95 °C. Cell extracts were obtained after centrifugation to remove cell debris and magnetic beads. Protein concentration was determined by the Bradford method, and protein was derivatized with dinitrophenylhydrazine as published previously (28). In the case of chronological aging, cell extracts were prepared as described previously (29). Protein concentration was determined by the Bradford method, and protein was derivatized with dinitrophenylhydrazine as before.

Two-dimensional Gel Electrophoresis and Western Blot Analysis—Protein from cell extracts was precipitated with acetone (at room temperature to minimize SDS precipitation), rinsed three-times in acetone, and resuspended in rehydration buffer (9 m urea, 4% CHAPS, 50 mM dithiothreitol, 0.5% immobilized pH gradient buffer (Amersham Biosciences), and traces of bromphenol blue) at a final concentration of 2–4 mg/ml. Isoelectric focusing (10–30 μg of protein) was performed in immobilized pH gradient strips. Second dimension SDS-PAGE was performed on 11% acrylamide gels followed by anti-dinitrophenyl Western blot (30). Parallel gels were silver-stained to visualize total protein and were used for protein identification. Proteins were identified by fingerprint mass spectroscopy.

Enzyme Activities—Total cellular iron was determined under reducing conditions with bathophenanthroline sulfonate as chelator (29). Detection of thiobarbituric acid-reactive substances (TBARS) was carried out by means of a fluorimetric assay (31). Lipofuscin was detected by its broad spectrum of autofluorescence, but to simplify the figure, only one barrier filter image is shown (λex 450–490; λem 520 nm). Quantification of protein carbonyl content of crude extracts was performed by high-pressure liquid chromatography after dinitrophenylhydrazine derivatization (28). Values are given in nmol/mg protein. Resistance to H2O2 was tested on cells grown either on 2% glucose or 0.5% glucose. At the middle of exponential phase (A600 = 0.5), 5 mM H2O2 was added to the culture. After a 1-h treatment, viability was measured by plating serial dilutions (1:5) on YPD plates.

RESULTS

Calorie Restriction Prevents Protein Oxidative Damage Resulting from Replicative Aging—One of the most studied markers resulting from oxidative stress is protein carbonyl formation (30, 37–39). Although protein damage produced under
Oxidative Damage in Aged S. cerevisiae

Identification of major oxidized proteins in both replicative and chronologically aged cells

<table>
<thead>
<tr>
<th>Spot number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Replicative</th>
<th>Protein&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Spot number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Chronological</th>
<th>Protein&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ssa1 (Hsp70 family)</td>
<td>Stress resistance proteins</td>
<td>1</td>
<td>Hsc82 (Hsp90 family)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Ssa2 (Hsp70 family)</td>
<td>2</td>
<td>Hsp90 (Hsp90 family)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 – 5</td>
<td>Hsp90</td>
<td>3</td>
<td>Ssa1 (Hsp70 family)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Ssa2 (Hsp70 family)</td>
<td>4</td>
<td>Ssa2 (Hsp70 family)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Hsp60</td>
<td>5</td>
<td>Hsp60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 – 9</td>
<td>Catalase T</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 – 7</td>
<td>Pyruvate decarboxilase</td>
<td>Glucose and energy metabolism</td>
<td>6</td>
<td>Transketolase</td>
<td></td>
</tr>
<tr>
<td>8 – 10</td>
<td>Enolase 2</td>
<td>10 – 13</td>
<td>Pyruvate decarboxilase</td>
<td>ATP synthase (β subunit)</td>
<td></td>
</tr>
<tr>
<td>11 – 13</td>
<td>Fructose-1,6-bisphosphate aldolase</td>
<td></td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 – 16</td>
<td>Alcohol dehydrogenase I</td>
<td></td>
<td>20 – 23</td>
<td>Enolase 2</td>
<td></td>
</tr>
<tr>
<td>17 – 18</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td></td>
<td>24 – 26</td>
<td>Enolase 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fructose-1,6-bisphosphate aldolase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Alcohol dehydrogenase I</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cytoskeleton</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27 Actin</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Spot number refers to two-dimensional gels obtained from replicatively aged cells (Fig. 1).

<sup>b</sup> Proteins were identified by fingerprint mass spectrometry. Proteins identified in both types of aging are in boldface type.

<sup>c</sup> Spot number refers to two-dimensional gels obtained from chronologically aged cells (Fig. 2). Spots 15 – 16 refer to ORF Ymr 323wp, spot 17 refers to ORF Ylr044wp, and spots 18 – 19 refer to ORF Ylr252wp.

Except for the mitochondrial Hsp60, the main oxidized proteins of both replicative and chronological-aged cells shared most of the oxidized proteins. Hsp70 and the mitochondrial Hsp60 were common targets, but Hsp90s (both the inducible Hsp82 and the constitutive Hsc82) and catalase T were only present as damaged proteins under chronological aging. The enzymes from the glycolytic and the energy metabolism pathways became highly oxidized in both models.

Enolase 1 is highly induced after the diauxic shift and present in equal amounts along the stationary phase compared with enolase 2. This isoenzyme was more prone to oxidation than enolase 1, which could only be detected after day 23 (Fig. 2A). It is also interesting to observe that several major spots were clearly resistant to oxidation (Fig. 2C). Those spots are indicated in the legend of Fig. 2. Calorie restriction also prevented oxidative damage on budding aging (Fig. 2D). A time course of protein oxidation was quantitated by high pressure liquid chromatography (Fig. 2E). A clear increase was observed in cells grown at high glucose concentration; meanwhile, cells grown with 0.5% glucose showed lower and stable levels of protein carbonyls.

Caloric Restriction Prevents Several Phenotypic Characteristics of Aging and Increases Chronological Life Span—In addition to protein oxidation, we analyzed several phenotypic traits (iron, lipofuscin, and TBARS) on chronologically aged yeast grown either on 2 or 0.5% glucose. Iron is one of the major agents involved in oxidative damage, which through the Fenton reaction produces the highly reactive hydroxyl radical. As shown in Fig. 3A, there was a 4 – 5-times increase of iron accumulation in cells grown on 2% glucose. In these cells, lipofuscin accumulation, which has been known for many years as a hallmark of aging in higher organisms (40), also was clearly visible (Fig. 3B). Its broad fluorescent spectrum has been described as indicative of this insoluble mixture of lipids, proteins, and nucleic acids, which present additionally high concentrations of metals, especially iron (40). Lipid peroxidation measured as TBARS showed a straight increase over time in cells grown on 2% glucose, reaching (after 40 days) values 8 – 10 times higher than those observed in exponentially growing cells (Fig. 3C). These aging markers were greatly reduced under caloric restriction (Fig. 3, A – C).

The results described above are in agreement with the differences in cell viability observed over the stationary phase (Fig. 3D). Under our conditions, <5% of the cells remained viable when grown in YPD with 2% glucose after 60 days in culture. After the same period, >90% of the cells were viable when cultured in YPD with 0.5% glucose. Calorie restriction also increased survival when synthetic medium was used instead of YPD (Fig. 3E). The increase in viability could be explained by an improved resistance to oxidative stress. Increased respiration on low glucose would trigger the synthesis of antioxidant systems. In agreement with this assumption, the
results shown in Fig. 3F indicated that calorie-restricted cells presented higher resistance to \( \text{H}_2\text{O}_2 \).

**Enzyme Activities Are Differentially Affected by Aging and Calorie Restriction**—The relevance of protein oxidation in enzyme activity could give a clue of its role in cell viability. The activity of the main oxidatively damaged enzymes was compared among cells cultured on 2 or 0.5% glucose from exponential to late stationary phase (60 days). As is shown in Fig. 4, the evolution of catalase activity was clearly different among cells grown on 2 and 0.5% glucose. While the activity remained high in 0.5% glucose, a progressive decrease starting around day 20 was observed in the 2% glucose. Interestingly, this inactivation was concomitant with its carbonylation (Fig. 4). The difference observed in catalase activity prompted us to study SOD, another antioxidant enzyme, especially relevant in stationary phase (41). There was a well known increase in both cytosolic and mitochondrial SOD activities during the diauxic shift. In stationary phase, a greater decrease was observed in cells grown on 0.5% glucose, a progressive decrease starting around day 20, reaching a minimum level at day 60, versus a greater decrease in cells grown on 2% glucose. Interestingly, this decrease was concomitant with its carbonylation (Fig. 4).

Activities of the glycolytic enzymes showed a decrease starting around day 30, reaching a minimum level at day 60, in accordance with maximum protein oxidation. During this period, no differences in protein levels, measured by Western blot, were found (data not shown). The lack of correlation between increase in protein carbonylation and maintenance of enzyme activities at the beginning of stationary phase may be explained by synthesis of isoenzymes with different physiological functions to those present in exponential phase. However, no relevant differences between restricted versus non-restricted cells were observed at the end of the stationary phase (Fig. 4).

**DISCUSSION**

The results described in this paper showed that oxidative damage found in both replicative and chronological-aged yeast presented clear differences between cells grown on 2% and cells grown on 0.5% glucose. In chronological aging, the metabolic change occurring along the diauxic shift triggered a huge induction of stress defenses. As a result, cells in stationary phase are highly resistant to several stresses including oxidative stress (42, 43). Nevertheless, there is a ROS increase observed in old cells grown at high glucose concentration. ROS formation may be a consequence of the iron accumulation throughout the stationary phase. Iron is a transition metal essential in redox reactions, but this property makes it potentially toxic. Lipofuscin, which contains high amounts of this metal, also accumulates in this phase. This often called age pigment is an intralysosomal, insoluble polymeric substance primarily composed of cross-linked protein residues and lipids and is formed because of iron-catalyzed oxidative processes (40). Because iron accumulated in lipofuscin is not available for the cells, this situation could be sensed as an intracellular metal depletion and iron transport system would be induced to promote entrance of iron. Consequently, ROS generation will increase and damage to all cellular macromolecules will occur.

Protein carbonyl detection as a measure of oxidative damage revealed that two important groups of proteins became modified in this situation: 1) enzymes of the glycolytic pathway and 2) molecular chaperones. Interestingly, these targets were already identified as oxidatively modified proteins by treatment of yeast cells with \( \text{H}_2\text{O}_2 \) (30). Under such stress, major targets such as enolase and glyceraldehyde-3-phosphate dehydrogenase were highly inactivated because of oxidative modification. Beside the susceptibility of glycolytic enzymes to oxidation...
during aging, an interesting observation is the different oxidative modification of enolase isoenzymes. Although enolase 2 involved in glycolytic flux appears to be one of the first oxidized proteins, enolase 1 expressed in the post-diauxic shift and involved in gluconeogenesis showed a marked resistance to oxidation. Whether this means that an evolutionary adaptation to stressing conditions occurs in yeast cells as they age (24, 44) will deserve future investigations.

It has been described that replicative aged cells show markers of oxidative stress (24, 44). The results presented here obtained from a proteomic analysis revealed that oxidatively modified proteins in replicative aged cells were, interestingly, highly similar to those we found in chronological aging. Five enzymes of the glycolytic pathway were oxidatively damaged. How can such a modification be interpreted with respect to life span? An inactivation of glycolytic enzymes will lead to the impairment of glucose utilization, which correlates with the enhancement of the gluconeogenic and energy storage pathways observed during replicative aging (45). In addition, reducing glycolysis has proved to increase longevity in several organisms (46). Therefore, de-
creased activity of these enzymes on high glucose cultures would act in favor of life span lengthening. Molecular chaperones are involved on protein translocation, folding, and assembly, and their expression is induced in several stresses (47). Our results showed that Hsp60, Hsp70s, and Hsp90s became carbonylated under replicative and/or chronological aging. It is reasonable to assume that such modifications would alter their chaperone activity, thus affecting cell survival. This assumption is based on the fact that mutants of mitochondrial Hsp60 are not viable (48) and that increased expression of Hsp60 confers a higher resistance to oxidative stress (29). Also, mutations on Hsc22 results in a decrease of chronological life span (49).

Our results also showed that, in chronological aging, actin appeared as a carbonylated protein. Aguilaniu et al. (24) propose that actin skeleton is required for proper segregation of oxidized proteins during cytokinesis. This hypothesis is based on both the inhibition of actin assembly by latrunculin A and the atypical spatial distribution of actin in a Δsir2 mutant. In each case, retention of oxidized proteins by mother cells was abolished. Based on these results, oxidative modification of actin provides some clues to explain why replicative life span decreased after passage through the stationary phase (50).

Calorie restriction has been described to extend life span in several organisms (19), including replicative life span in yeast (21). With respect to high glucose, the main metabolic change is a shift from fermentation to respiration. The increase in mitochondrial activity then would trigger the formation of ROS, and consequently, antioxidant defenses would be induced. Such mild stress may promote a better adaptation to ROS, explaining the decreased oxidative damage observed in calorie-restricted aged cells (both replicative and chronological). Consistently, resistance to H$_2$O$_2$ was higher in cells grown on 0.5% glucose with respect to those grown on 2% glucose. In replicative aging, the reduction of protein damage in 18-generation-old yeast grown on 0.5% glucose was especially relevant for Hsps (Fig. 1). This reduced oxidative damage may work synergically with Sir2p, which is crucial for understanding the aging of post-mitotic cells. One of the important results described in this paper refers to the altered regulation of antioxidant genes in catalase-deficient cells. It has been reported that overexpression of SOD2 increased SOD1 and catalase activities as a consequence of H$_2$O$_2$ production (52). It has been demonstrated that SOD1 and SOD2 are essential for stationary phase survival, especially the cytosolic isozyme (12, 41). In accordance with the increased life span of calorie-restricted cells, higher levels of cytosolic SOD activity were found in cells at late stationary phase compared with non-restricted ones.

The results described in this paper provide evidence that both aging models can be explained, at least in part, by physiological changes triggered by oxidative damage to specific proteins produced by a stress situation inside the cell. The success of calorie restriction slowing down aging may be attributed to a better preparation of young cells to cope with increased stress situation. This would keep molecular chaperones catalase and SOD functional as the cells grow old.

Acknowledgments—We thank Vanessa Guijarro for excellent technical assistance. We are indebted to Rodney L. Levine for critical review of the paper and helpful suggestions.

REFERENCES

Oxidative Damage to Specific Proteins in Replicative and Chronological-aged Saccharomyces cerevisiae: COMMON TARGETS AND PREVENTION BY CALORIE RESTRICTION

Gemma Reverter-Branchat, Elisa Cabiscol, Jordi Tamarit and Joaquim Ros

doi: 10.1074/jbc.M404849200 originally published online May 27, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404849200

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 49 references, 23 of which can be accessed free at
http://www.jbc.org/content/279/30/31983.full.html#ref-list-1