Antioxidants: Redefining Their Roles

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The cellular environment is sensitive to the presence of free radicals, which are molecules with unpaired electrons. The most common types of free radicals are formed from the elements oxygen, nitrogen, carbon, sulfur, and chlorine. Cells continually need to balance redox potential (the tendency to gain or lose electrons). This potential can be skewed toward oxidation (a tendency to lose electrons), called oxidative stress, or reduction (a tendency to gain electrons), called reductive stress.

In the past, oxidative stress was used as a general term to describe damage to cells, tissues, and organs by reactive oxygen species (ROS) or reactive nitrogen species (RNS). ROS include the superoxide radical (O_2-), peroxyl radicals, hypohalite radicals, hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH-). Reactive nitrogen species include nitric oxide radicals (NO), peroxynitrite radicals (ONOO-), and other organic molecules modified by these pro-oxidant chemical species.[1]

At physiological levels, free radicals play important positive roles in cellular physiology, including cell signaling and proper immune function.[2] However, free radicals can also damage all classes of macromolecules (eg, lipids, proteins, nucleic acids), and excess free radicals have been implicated in the pathogenesis of more than 100 disorders,[3] including atherosclerosis, cancer, diabetes, Alzheimer's disease, rheumatoid arthritis, and inflammatory bowel disease.[3-9]

Opposing free radicals are antioxidants. According to the classical definition, these molecules—which include vitamins A, C, and E; glutathione; alpha lipoic acid; and many others—can donate an electron, thereby reducing the free radical and restoring its stability. This "quenches" the free radical and stops it from doing additional damage to other molecules. For example, the mechanism by which vitamin E maintains cellular membrane integrity is through its free-radical quenching potential.[10] However, once the antioxidant has donated an electron, it becomes a free radical itself and must be reduced by another molecule through accepting an electron. The biological difficulty of controlling free radical propagation merely through an unending sequence of electron donors is improbable. Simply put, this explanation seems too linear for biological systems in which biochemical pathways are interrelated. Hence, it seems plausible other mechanisms are at work.

Although accurate in many important respects, this classical understanding of antioxidants as electron donors quenching damaging free radicals in the cytosol ignores perhaps the most vital roles these molecules play in the body. What this definition does not take into account is the fact that, through means other than directly quenching free radicals, these compounds also modulate cellular redox potential and cellular physiology by directly altering cell signaling and transcription. Under this broader concept of "redox molecules," use of antioxidant therapy in clinical practice might now be more accurately viewed as targeting specific signal transduction proteins, transcription factors, and genes, in addition to quenching free radicals.

Redox-sensitive pathways

The redox potential within cells influences the cell's regulation of transcription factors, signaling pathways, and ultimate survival (Table 1).[11] For example, when the

Table 1. Effects of Redox Molecules on Signaling Pathways

Inhibition of	By redox molecule
5-lipoxygenase	Caffeic acid[21]
COX-2	Vitamin E,[21,22,24] kaempferol,[21] quercetin,[21] genistein,[21] resveratrol[21]
IL-1 mediated signaling	Curcumin[33]
iNOS expression induced by bacterial LPS	Melatonin[34]
NF-κβ	Vitamin C,[11,25] curcumin,[27,35,36] glutathione,[12] beta-carotene,[11] N-acetylcysteine,[37] alpha lipoic acid,[37,38] selenium,[11] epigallocatechin gallate,[21,39-41] quercetin[42]
Protein kinase C	Caffeic acid,[21] vitamin E,[20] alpha-lipoic acid,[20] vitamin C[20]
TNF-α production	Silymarin,[43] curcumin[44]
Tyrosine phosphorylation of Syk	Quercetin[30]

redox potential favors oxidation, nuclear factor-kappa B (NF-κβ), a pro-inflammatory transcription factor, is upregulated—producing pro-inflammatory molecules including interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and inducible nitric oxide synthase (iNOS).[12,13] Excessive NF-κβ activation and inflammation are implicated in many conditions, including the aging process and neurodegenerative diseases.13 Along with free radicals, numerous chemical signals can also cause NF-κβ upregulation, including lipopolysaccharides (LPS) from gram-negative bacteria such as *Escherichia coli* and cytokine tumor necrosis factor alpha (TNF-α).[12-14]

Activation of specific enzymes and their metabolites can also cause pro-oxidant states. Cyclooxygenase (COX) and 5-lipoxygenase (5-LOX), for example, are key enzymes in the biosynthesis of prostaglandins and leukotrienes from arachidonic acid, a 20-carbon polyunsaturated fatty acid of the omega-6 series (20:4 n-6). Two major isoforms of the COX enzyme exist: COX-1 and COX-2. Specifically, phospholipase A2, an enzyme that cleaves arachidonic acid from its glycerol backbone in cell membranes, frees arachidonic acid to enter the COX or LOX pathway. COX-1 is constitutively expressed, while COX-2 is inducible. COX-2 expression is increased in vitro by various reactive oxygen intermediates including IL-1, TNF-α, LPS, superoxide radicals, and hydrogen peroxide.[15] Increased expression of COX-2 has been implicated in inflammation-mediated pathologies, such as Alzheimer's disease and cancer.[16,17] Leukotriene B4 (LTB4), a metabolite of the LOX pathway, is involved in the pathogenesis of asthma.

Another pro-oxidant system is the nicotinamide adenine dinucleotide (NADH)-oxidase enzyme, which produces free radicals[18] and has also been shown to increase COX-2 expression in vitro.[15] In rats, inhibition of NADH-oxidase prevents alcohol-induced liver damage and fibrosis.[19]

Oxidative stress can also directly stimulate cell-signaling pathways that are upregulated in many different diseases. Protein kinase C (PKC) is such a pathway. According to C.A. Carter and C.J. Kane, who wrote an excellent review of PKC biochemistry and its influence on health and disease, PKC "is involved in intracellular signal transduction pathways that regulate gene transcription, differentiation, cell cycle, cytoskeletal functions, apoptosis, growth factor response, cell-cell interaction, cell migration, senescence, and drug resistance."[20]

Oxidized low-density lipoprotein (OxLDL) cholesterol uptake by macrophages activates PKC, which is an important step in the pathogenesis of atherosclerosis. PKC causes muscle insulin resistance and may contribute to the development of diabetes. Also, according to Carter and Kane, "increased PKC activity has been unequivocally associated with carcinogenesis and tumor metastasis."[20] A number of antioxidants have been shown in vitro to inhibit PKC activity, including vitamin E, vitamin C, and alpha lipoic acid,[20] as well as caffeic acid.[21]

Redox molecules

A number of redox molecules have been shown in vitro to inhibit NF-β, phospholipase A2, COX, LOX, iNOS, TNF-α, NADH, and PKC activity, including vitamin E, caffeic acid, alpha lipoic acid, curcumin, and melatonin (see Table 2).

Vitamin E: This vitamin provides an example of how the antioxidant concept has broadened with recent research. Vitamin E is a lipid-soluble vitamin first described in 1922 by H. McLean Evans and Katharine Scott Bishop as an essential nutrient for rat reproduction.[22] For 60 years, the actions of vitamin E have typically been ascribed to its ability to scavenge free radicals and, in particular, protect lipid membranes from damage.[10,22,23] However, most of the nutrient's activities in vivo may actually result from its ability to "modulate cellular behavior by specific interactions with enzymes, structural proteins, lipids, and transcription factors."[22] Although it has been postulated that these effects occur by mechanisms other than simple free radical quenching, the exact mechanisms have yet to be fully defined.[22]

Among these roles, α-tocopherol, a vitamin E analogue, has been shown to inhibit phospholipase A_2 and COX-2[24] as well as the assembly of the multi-protein NADPH-oxidase complex.[22] Less is known about the actions of vitamin E's other tocopherols and tocotrienols. However, it can be assumed that they also likely influence the redox potential of cells by altering intracellular mechanisms.

The wide-ranging effects of vitamin E on cellular physiology should be viewed as the rule and not the exception among antioxidants. Antioxidants appear to do more than simply scavenge free radicals, and as the details of their mechanisms are clarified, the use of targeted antioxidant therapy in clinical settings should increase.

Vitamin C: In another example, the classic view of vitamin C has been that it donates an electron and therefore quenches ROS. However, vitamin C is not merely a free-radical quencher. Ex vivo and in vitro experiments determined that reduced and oxidized vitamin C inhibit NF–κβ, thereby decreasing inflammation.[25] Reduced vitamin C exerts this action by quenching free radicals, while oxidized vitamin C directly inhibits inhibitory kappa kinase (IKK) activity.[25] IKK is a multimeric protein that inhibits NF-κβ activation. The phosphorylated IKK subunit Iκβα releases NF-κβ, allowing it to migrate into the nucleus and initiate transcription of pro-inflammatory compounds such as TNF-α.

Flavonoids: Found in many plants, flavonoids are considered antioxidants. As redox molecules, curcumin

TABLE 2. NON-VITAMIN REDOX MOLECULES

Antioxidant	Description	Good Sources
Alpha lipoic acid	Both lipid- and water-soluble. Involved in energy production pathways and may increase glutathione levels.	Food sources of lipoic acid have not been calculated, but have been reported in appreciable quantities in liver (1.3 mcg/g dry weight), spinach, and broccoli.[45]
Beta-carotene	Precursor to vitamin A.	Yellow, orange, red, and leafy green vegetables ; and fruits (eg, carrots, tomatoes, sweet potatoes, winter squash , broccoli, spinach, lettuce varieties , and cantaloupe).
Caffeic acid	A carboxylic acid found in many fruits, vegetables, seasonings, and beverages.	Dandelion (*Taraxacum officinale*), yarrow (*Achillea millefolium*), horsetail (*Equisetum spp*.), among others.
Curcumin	A polyphenol found in the spice turmeric.	Turmeric (*Curcuma longa*).
Epigallocatechin gallate (EGCG)	A catechin (class of tannins), with antioxidant activity about 25-100 times that of vitamins C and E.	Green tea (*Camellia sinensis*).
Genistein	An isoflavone (class of flavonoids) with phytoestrogenic activities.	Soybeans and soy foods.
Glutathione	A tripeptide formed from the amino acids L-glutamine, L-cysteine, and glycine. The major endogenous antioxidant.	N-acetylcysteine, lipoic acid, curcumin, and whey protein all increase endogenous glutathione.
Kaempferol	A flavonoid, found in many plants, that is yellow when isolated.	Apples, onions, leeks, citrus fruits, grapes, red wine, *Gingko biloba*, St. John's wort (*Hypericum perforatum*).
Melatonin	Hormone secreted by the pineal gland. Involved in regulating circadian rhythms.	Produced endogenously from tryptophan. Requires vitamin B-6.
N-Acetylcysteine (NAC)	Antioxidant and mucolytic. Increases endogenous glutathione. In high doses, used to treat paracetamol (acetaminophen) toxicity.	Food is not a significant source of NAC. Available as a dietary supplement.
Quercetin	A flavonoid found in many plants.	Apples, black tea, green tea, onions, raspberries, red wine, red grapes, citrus fruits, broccoli, leafy green vegetables, cherries.
Resveratrol	A polyphenolic phytoalexin, produced by plants as an antifungal agent.	Skin of red grapes.
Selenium	A trace mineral.	Best sources are plants. Also found in smaller quantities in animal foods. Content of selenium in plants depends on the selenium content of the soil in which they were grown. Brazil nuts, one of the best sources of selenium, contain approximately 545 mcg selenium per ounce of nuts.
Silymarin	A mixture of flavolignans (silybin, silydianin, silycristin) that increases endogenous glutathione.	Milk thistle (*Silybum marianum*)

from turmeric (*Curcuma longa*) and epigallocatechin gallate (EGCG) from green tea (*Camellia sinensis*) affect cell signaling. Curcumin and EGCG suppress constitutive and inducible NF-κβ activation in vitro.[26]

Curcumin altered cellular redox potential in alveolar epithelial cells by directly quenching superoxide anions and hydroxyl radicals, and also indirectly by stimulating the synthesis of glutathione and by inhibiting TNF-α- and H_2O_2-induced NF–κβ binding to DNA.[27] A recent pilot study of high-dose curcumin (1440–1560 mg/d) for 2 to 3 months in volunteers with irritable bowel disease significantly reduced inflammation compared to baseline, and

also improved subjective and other objective measurements.[28] In vitro data supports multiple explanations for curcumin's anti-inflammatory activity. On the one hand, curcumin is a powerful antioxidant that scavenges free radicals; however, curcumin also increases glutathione synthesis by upregulating the expression of γ-glutamylcysteine ligase catalytic subunit (GCLC) and IL-8 gene expression.[27] Curcumin's ability to modulate signaling pathways is a more complete explanation for its anti-inflammatory effects than saying that it merely acts by donating an electron to quench free radicals.

Quercetin is a flavonoid present in high levels in onions (*Allium cepa*), apples (*Malus spp.*), tea, and wine. It is considered an antioxidant with important cardiovascular benefits. In vitro and in vivo research demonstrates that quercetin inhibits platelet aggregation by inhibiting tyrosine phosphorylation of important signaling molecules.[29,30] In one study, after a washout period of 14 days during which they consumed a low quercetin diet, 6 healthy volunteers (3 men and 3 women) consumed 150 mg or 300 mg quercetin-4'-O-β-D-glucoside (using a preparation called Q-4-G, made by Polyphenols Laboratories AS based in Sandnes, Norway), which is "found in onions at particularly high levels."[30] Thirty minutes after this single-dose ingestion of Q-4-G, total flavonoid levels (quercetin and two of its metabolites, isorhamnetin and tamarixetin) peaked at 4.66 μM (± 0.77) and 9.72 μM (± 1.38), respectively, indicating dose-dependent systemic availability.

Ex vivo analysis determined collagen-stimulated platelet aggregation was significantly inhibited in both groups. Whole blood analysis showed significant inhibition of collagen-stimulated tyrosine phosphorylation, an important step in the platelet aggregation signaling cascade (P= 0.001). Specifically, 150 mg and 300 mg of Q-4-G significantly decreased phosphorylation of the Syk protein by 41.5 ± 16.25% and 37.2 ± 11.32% after 120 minutes, respectively (P= 0.05). Syk is a tyrosine kinase-dependent signaling protein that, when phosphorylated, initiates a cascade of signaling events that promotes platelet aggregation,[30] activates immune cells,[31] and has been implicated in tumorogenesis.[32] Similarly, collagen-stimulated phosphorylation of phospholipase Cγ2 was inhibited by 45 ± 10.3% and 38 ± 6.5% after 120 minutes of ingesting 150 mg and 300 mg Q-4-G, respectively (P= 0.05).[30]

Conclusion

The term "antioxidant" refers to a class of compound that quenches free radicals; however, that is only part of the story. These molecules also modulate intracellular redox potential by affecting cell signaling and transcription. A more accurate term is "redox molecules." Vitamins such as C and E fit this new definition, as do flavonoids such as curcumin, quercetin, and EGCG. Understanding this new definition may help spur the development of novel treatments by targeting specific pathways using natural compounds.

References

1. Biswas S, Chida AS, Rahman I. Redox modifications of protein-thiols: emerging roles in cell signaling. *Biochem Pharmacol*. 2006;71(5):551-564.
2. Poli G, Leonarduzzi G, Biasi F, Chiarpotto E. Oxidative stress and cell signalling. *Curr Med Chem*. 2004;11(9):1163-1182.
3. Bandyopadhyay U, Das D, Banerjee R. Reactive oxygen species: Oxidative damage and pathogenesis. *Curr Sci*. 1999;77(5):658-666.
4. Dalton TP, Shertzer HG, Puga A. Regulation of gene expression by reactive oxygen. *Ann Rev Pharmacol Toxicol*. 1999;39(1):67-101.
5. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. *Am J Cardiol*. 2003;91(3A):7A-11A.
6. Maheshwari RK, Singh AK, Gaddipati J, Srimal RC. Multiple biological activities of curcumin: a short review. *Life Sci*. Mar 27 2006;78(18):2081-2087.
7. Puddu P, Puddu GM, Galletti L, Cravero E, Muscari A. Mitochondrial dysfunction as an initiating event in atherogenesis: a plausible hypothesis. *Cardiology*. 2005;103(3):137-141.
8. Schon EA, Manfredi G. Neuronal degeneration and mitochondrial dysfunction. *J Clin Invest*. 2003;111(3):303-312.
9. West IC. Radicals and oxidative stress in diabetes. *Diabet Med*. 2000;17(3):171-180.
10. Groff JL, Gropper SS. Chapter 10: The Fat-Soluble Vitamins. *Advanced Nutrition and Human Metabolism*. Third ed. Belmont, CA: Wadsworth; 2000:316-370.
11. McEligot AJ, Yang S, Meyskens FL, Jr. Redox regulation by intrinsic species and extrinsic nutrients in normal and cancer cells. *Annu Rev Nutr*. 2005;25(1):261-295.
12. Rahman I, Biswas SK, Jimenez LA, Torres M, Forman HJ. Glutathione, stress responses, and redox signaling in lung inflammation. *Antioxid Redox Signal*. 2005;7(1-2):42-59.
13. McCann SM, Mastronardi C, De Laurentis A, Rettori V. The nitric oxide theory of aging revisited. *Ann NY Acad Sci*. 2005;1057(1):64-84.
14. Wheeler DS, Catravas JD, Odoms K, Denenberg A, Malhotra V, Wong HR. Epigallocatechin-3-gallate, a Green Tea-Derived Polyphenol, Inhibits IL-1{beta}-Dependent Proinflammatory Signal Transduction in Cultured Respiratory Epithelial Cells. *J Nutr*. 2004;134(5):1039-1044.
15. Feng L, Xia Y, Garcia GE, Hwang D, Wilson CB. Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-alpha, and lipopolysaccharide. *J Clin Invest*. 1995;95(4):1669-1675.
16. Pasinetti GM, Aisen PS. Cyclooxygenase-2 expression is increased in frontal cortex of Alzheimer's disease brain. *Neuroscience*. 1998;87(2):319-324.
17. Shishodia S, Aggarwal BB. Cyclooxygenase (COX)-2 inhibitor celecoxib abrogates activation of cigarette smoke-induced nuclear factor (NF)-kappaB by suppressing activation of IkappaBalpha kinase in human non-small cell lung carcinoma: correlation with suppression of cyclin D1, COX-2, and matrix metalloproteinase-9. *Cancer Res*. 2004;64(14):5004-5012.
18. Kono H, Rusyn I, Yin M, et al. NADPH oxidase-derived free radicals are key oxidants in alcohol-induced liver disease. *J Clin Invest*. 2000;106(7):867-872.
19. Kono H, Rusyn I, Uesugi T, et al. Diphenyleneiodonium sulfate, an NADPH oxidase inhibitor, prevents early alcohol-induced liver injury in the rat. *Am J Physiol Gastrointest Liver Physiol*. 2001;280(5):G1005-G1012.
20. Carter CA, Kane CJ. Therapeutic potential of natural compounds that regulate the activity of protein kinase *C. Curr Med Chem*. 2004;11(21):2883-2902.
21. Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI, Bahorun T. Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mutat Res*. 2005;579(1-2):200-213.
22. Zingg JM, Azzi A. Non-antioxidant activities of vitamin E. *Curr Med Chem*. 2004;11(9):1113-1133.
23. Ricciarelli R, Zingg JM, Azzi A. Vitamin E 80th anniversary: a double life, not only fighting radicals. *IUBMB Life*. 2001;52(1-2):71-76.

24. Wu D, Hayek MG, Meydani S. Vitamin E and macrophage cyclooxygenase regulation in the aged. *J Nutr*. 2001;131(2):382S-388S.
25. Carcamo JM, Pedraza A, Borquez-Ojeda O, Zhang B, Sanchez R, Golde DW. Vitamin C is a kinase inhibitor: dehydroascorbic acid inhibits IkappaBalpha Kinase beta. *Mol Cell Biol* 2004;24(15):6645-6652.
26. Sarkar FH, Li Y. Cell signaling pathways altered by natural chemopreventive agents. *Mutat Res*. 2004;555(1-2):53-64.
27. Biswas SK, McClure D, Jimenez LA, Megson IL, Rahman I. Curcumin induces glutathione biosynthesis and inhibits NF-kappaB activation and interleukin-8 release in alveolar epithelial cells: mechanism of free radical scavenging activity. *Antioxid Redox Signal*. 2005;7(1-2):32-41.
28. Holt PR, Katz S, Kirshoff R. Curcumin therapy in inflammatory bowel disease: a pilot study. *Dig Dis Sci*. 2005;50(11):2191-2193.
29. Ferry DR, Smith A, Malkhandi J, et al. Phase I clinical trial of the flavonoid quercetin: pharmacokinetics and evidence for in vivo tyrosine kinase inhibition. *Clin Cancer Res*. 1996;2(4):659-668.
30. Hubbard GP, Wolffram S, Lovegrove JA, Gibbins JM. Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans. *J Thromb Haemost*. 2004;2(12):2138-2145.
31. Sada K, Takano T, Yanagi S, Yamamura H. Structure and function of Syk protein-tyrosine kinase. *J Biochem* (Tokyo). 2001;130(2):177-186.
32. Stewart Z, Pietenpol J. Syk: a new player in the field of breast cancer. *Breast Cancer Res.* 2001;3(1):5-7.
33. Jurrmann N, Brigelius-Flohe R, Bol G-F. Curcumin blocks interleukin-1 (IL-1) signaling by inhibiting the recruitment of the IL-1 receptor-associated kinase IRAK in murine thymoma EL-4 Cells. *J Nutr*. 2005;135(8):1859-1864.
34. Aydogan S, Yerer MB, Goktas A. Melatonin and nitric oxide. *J Endocrinol Invest*. 2006;29(3):281-287.
35. Jobin C, Bradham CA, Russo MP, et al. Curcumin blocks cytokine-mediated NF-kappa B activation and proinflammatory gene expression by inhibiting inhibitory factor I-kappa B kinase activity. *J Immunol*. 1999;163(6):3474-3483.
36. Singh S, Aggarwal BB. Activation of transcription factor NF-kappaB is suppressed by curcumin (diferuloylmethane). *J Biol Chem*. 1995;270(42):24995-25000.
37. Smith AR, Shenvi SV, Widlansky M, Suh JH, Hagen TM. Lipoic acid as a potential therapy for chronic diseases associated with oxidative stress. *Curr Med Chem*. 2004;11(9):1135-1146.
38. Packer L. Alpha-lipoic acid: a metabolic antioxidant which regulates NF-kappa B signal transduction and protects against oxidative injury. *Drug Metab Rev*. 1998;30(2):245-275.
39. Ahmad N, Gupta S, Mukhtar H. Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor kappaB in cancer cells versus normal cells. *Arch Biochem Biophys*. 2000;376(2):338-346.
40. Khan N, Afaq F, Saleem M, Ahmad N, Mukhtar H. Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate. *Cancer Res*. 2006;66(5):2500-2505.
41. Afaq F, Adhami VM, Ahmad N, Mukhtar H. Inhibition of ultraviolet B-mediated activation of nuclear factor kappaB in normal human epidermal keratinocytes by green tea constituent (-)-epigallocatechin-3-gallate. *Oncogene*. 2003;22(7):1035-1044.
42. Dias AS, Porawski M, Alonso M, Marroni N, Collado PS, Gonzalez-Gallego J. Quercetin decreases oxidative stress, NF-{kappa}B activation, and iNOS overexpression in liver of streptozotocin-induced diabetic rats. *J Nutr*. 2005;135(10):2299-2304.
43. Zi X, Mukhtar H, Agarwal R. Novel cancer chemopreventive effects of a flavonoid antioxidant silymarin: inhibition of mRNA expression of an endogenous tumor promoter TNF alpha. *Biochem Biophys Res Commun*. 1997;239(1):334-339.
44. Chan MM. Inhibition of tumor necrosis factor by curcumin, a phytochemical. *Biochem Pharmacol*. 1995;49(11):1551-1556.
45. Higdon J. Lipoic Acid. Micronutrient Information Center [web page]. April 10, 2006; http://lpi.oregonstate.edu/infocenter/othernuts/la/. Accessed August 8, 2006.