Published in final edited form as:

Methods Mol Biol. 2008 ; 447: 185–197.

Alcohol-Induced Oxidative Stress in the Liver:

In Vivo Measurements

Gavin E. Arteel

Summary

Oxidative stress is increasingly suspected to contribute to the initiation and progression of many disease, including those caused by alcohol exposure. Two major products of reactive oxygen and nitrogen species formation are 4OH-nonenal and 3-nitrotyrosine protein adducts, both of which can be detected by immunohistochemistry. In the past, immunohistochemical techniques have served largely as qualitative measures of changes. However, coupled with digital capture and analysis of photomicrographs, one can now quantitate treatment-related changes with immunohistochemistry. This chapter summarizes techniques for immunohistochemical detection of these products of reactive oxygen and nitrogen species and subsequent image-analysis. Although the methods described herein are based on liver, these techniques have been employed successfully in most tissue types with minor modifications and are therefore broadly applicable.

Keywords

Oxidative stress; immunohistochemistry; 4-hydroxynonenal; 3-nitrotyrosine; alcoholic liver disease

1 Introduction

Early free radical researchers were for the most part chemists (e.g., Henry John Horstman Fenton of the “Fenton” reaction; see (1) for review). However, free radical formation was considered for the most part an *ex vivo* phenomenon. Gershan and Gilbert (2) first proposed that radiation-induced cell killing might be mediated by oxygen free radicals, based on the observation that the pattern of toxicity of radiation was quite similar to that hyperoxygen in *vivo*. In the same year, Harman (3) published the “free radical hypothesis of aging.” This strength of this hypothesis can be demonstrated by the simple fact that it has endured for almost 50 yr. It was generally thought that free radical formation in the cell was limited to either external stimuli (such as radiation), or random events and did not occur under “normal” circumstances.

A major paradigm shift in free radical research came in 1969, when Fridovich and McCord described the function of SOD as a catalytic reducer of O_2^- to H_2O_2 (4). On the basis of these results, the concept that oxidants are produced by the cell under normal conditions gained hold. The discovery of enzymes that normally produce prooxidants (in contrast to electron leakage from other enzyme systems), such as NAD(P)H oxidases, xanthine oxidases, and myeloperoxidases, further strengthened the case that prooxidants are regularly found in *vivo*. Discoveries of catalytic functions of the glutathione peroxidases, glutaredoxin peroxidases, thioredoxin peroxidases, and catalases indicated that hydroperoxides are also kept in close check in *vivo*. It became clear that balance between prooxidants and antioxidants is critical for survival and functioning of aerobic organisms. An imbalance favoring prooxidants and/or disfavoring antioxidants, potentially leading to damage, was coined as oxidative stress by Sies (5).

Reactive oxygen and nitrogen species (ROS and RNS, respectively) can be products of normal cellular metabolism and have potentially beneficial effects (e.g., cytotoxicity against invading bacteria); indeed, there are enzymes in which the key function is to produce ROS/RNS, such as nitric oxide synthases (NOS), NA(D)PH oxidases, and myeloperoxidases. Oxidative stress is proposed to be critically involved in numerous diseases, including alcohol-induced liver disease. In 1966, DiLuzio was the first to characterize lipid peroxidation after chronic exposure to alcohol (6), a finding confirmed by others (7). After the introduction of the spin trapping technique, *in vitro* (8-10) and *in vivo* (11), evidence for a free radical derived from ethanol, the α -hydroxyethyl free radical was presented.

A general problem of studying prooxidants *in vivo* is that, because of their inherent reactivity, they generally cannot be measured directly. One is therefore often left with measuring products of the reaction of these molecules with other molecules (e.g., oxidative or nitrative modification of proteins). This indirect detection of “footprints” of ROS/NOS makes it difficult to conclusively identify the parent oxidant. However, coupled with *in vivo* modulation of free radical production, one can obtain relatively clear data supporting the involvement of specific free radical species involvement in alcohol-induced tissue damage.

This chapter will summarize some techniques that have been used in the study of oxidative stress in alcohol-induced liver injury. Immunohistochemical detection of 4OH-nonenal- and 3-nitrotyrosine-modified proteins will be detailed (12). In the past, immunohistochemical techniques have served largely as qualitative measures of changes but have the advantage of illuminating the location and zonation of the haptene of interest. However, with the advent of digital photography and image analysis, one can now quantitate treatment-related changes in the tissue of interest. Other advantages of these techniques are that they are readily performed in most basic research laboratories and do not require pretreatment of the animals to attain a signal. The latter point implies that historical samples may be analyzed. Although the methods described herein are based liver, these immunohistochemical techniques have been employed successfully in most tissue types with minor modifications.

Although immunohistochemical analysis of ROS/RNS-modified proteins is a useful technique, careful attention must be paid to minimize the effect of variability and artifacts. Obvious possible sources of *in vivo* variability (e.g., gender, age, dose, etc) should clearly be avoided. Furthermore, variability caused by the processing and staining of the tissue itself should also be minimized. This *ex vivo* variability is attributable predominantly to the staining itself; it is therefore imperative that all samples to be analyzed are stained simultaneously to avoid interassay inconsistencies. Reasonable measures should also be taken to avoid all possible sources of *ex vivo* variability, including harvesting and processing of the tissue for immunohistochemistry (*see* Note 1).

2 Materials

All solutions should be of analytical grade or purer. Nanopure water is used for preparing all solutions. Glassware should be freed of possible detergent residue by prerinsing with nanopure water.

2.1 Generation of an In Vivo Positive Control for Staining

1. Laboratory safety equipment (gloves, mask, protective eyewear, etc.)

¹To decrease *ex vivo* variability, our group always harvests sections for immunohistochemistry from the same liver lobes (left lateral and right medial) and the same location with the lobe (transverse section from the middle of the lobe) to avoid any potential geographic variation in the haptene signals.

2. Lipopolysaccharide (LPS) from *Escherichia coli* (O55:B5; Sigma).
3. Sterile saline: 0.9 % sodium chloride.
4. Needles for injection.
5. Borosilicate glass tubes (12 × 75 mm; Fisher).

2.2 Harvesting of Tissues for Immunohistochemistry

1. Laboratory safety equipment (gloves, mask, protective eyewear, etc.)
2. Rodent anesthetic (e.g., sodium pentobarbital, ketamine/xylazine, etc.).
3. Tools for harvesting tissue.
4. Sterile saline.
5. 10% neutral-buffered formalin (Fisher).
6. 10-mL syringes with plastic infusion needles (16 gage or larger).
7. Razor blades.
8. Plastic tissue cassettes.

2.3 Immunohistochemistry

1. Laboratory safety equipment (gloves, mask, protective eyewear, etc.)
2. Ventilated work area (e.g., chemical fume hood).
3. Magnetic stir plate capable of maintaining a slow stir rate (< 100 rpm) and a 1-inch magnetic stir bar (Fisher).
4. > 3× glass staining dishes (45 × 75 × 32 mm; “Coplin jars”) and slide holders (Wheaton).
5. Xylene (Fisher) or less toxic alternative (e.g., CitriSolv; Fisher).
6. 100% and 95% ethanol (Aaper).
7. Phosphate-buffered saline with 0.5% Tween-20 (PBS) (10× stock) 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄ (adjust to pH 7.4), and 5% Tween-20.
 - a. Autoclave before storage at room temperature. Sodium azide (0.02%) may be added to the stock to help prevent contamination. Prepare working solution by dilution of one part with nine parts water.
8. 10 mM sodium citrate (pH 6).
9. 30% hydrogen peroxide solution (Fisher).
10. Bovine serum albumin, fraction V (BSA; Sigma).
11. Clear nail polish or Dako Pen (Dako).
12. Slide rack (Fisher). A slide storage box (Fisher) works quite well for this purpose.
13. Rabbit polyclonal anti-4OH-nonenal antibody (Alpha Diagnostic); Rabbit polyclonal anti-nitrotyrosine antibody (Upstate).
14. EnVision, HRP-based staining system (Dako). It is not required, but this system usually gives a good signal to noise ratio and contains both the HRP-conjugated secondary antibody and DAB solutions. Alternatively, HRP-conjugated goat

antirabbit antibodies (Vector) and diaminobenzidine (DAB) solution (Dako) may be purchased separately.

15. Aqueous hematoxylin (Dako).
16. Mounting media (Permount; Fisher) and coverslips (25 × 50 × 0.15 mm; Fisher).

2.4 Digital Image Acquisition and Analysis System (see Note 2)

1. Microscope: Eclipse E600 with a 10× ocular Plan Fluor series objectives (4×, 10×, 20×, 40× and 100×) (Nikon)
2. Camera: Spot RT slider BW/color digital CCD camera (Diagnostic Instruments)
3. Imaging software: MetaMorph image-acquisition and analysis software (Molecular Devices; *see* Note 3).

3 Methods

Both staining protocols are relatively similar and have shared methods. The *in vivo* methods require prior approval by the local Institutional Animal Care and Use Committee (IACUC).

3.1 Generation of an *In Vivo*-Positive Control for 4OH-Nonenal and 3-Nitrotyrosine

It is important to have a positive *in vivo* control for the staining. In our experience, injections of hepatotoxic doses of LPS generate sufficient signals of both 4OH-nonenal and 3-nitrotyrosine to serve as positive controls. Fig. 1 contains representative photomicrographs (10×10 magnification) of a liver from a mouse 24 h after injection with LPS (10 mg/kg intraperitoneally) and immunostained for 4OH-nonenal. The supplier also recommends additional positive and negative controls for 3-nitrotyrosine staining. These can be generated by following manufacturer instructions.

1. Prepare an LPS-injectable solution in sterile saline (injection volume 1-2 mL/kg). Solution must be made in borosilicate glass tubes (LPS binds to plastic and Teflon). **Note:** LPS is highly toxic care should be taken when handling this compound to avoid exposure.
2. Inject the rodent (rat or mouse) with LPS intraperitoneally. This laboratory uses a dose of 10 mg/kg LPS (intraperitoneally) for both species. However, because of batch-to-batch variability in LPS potency, the dose should be titrated in the individual laboratory to achieve serum alanine aminotransferase (ALT) activity of 200-300 IU/L 24 h after LPS injection.
3. Harvest liver 24 h after LPS injection (*see* Subheading 3.2.).

3.2 Harvesting Tissue for Immunohistochemistry

1. Anesthetize the rodent according to your institution's IACUC-approved protocol.
2. Perform a laparotomy to fully-expose the liver.
3. Exsanguinate the animal by cutting the aorta just before entering the heart.

²There are many possible iterations of this system, but the emphasis should be on a system that is able to take high-quality and -resolution brightfield images. Important features of the system include flat-field correction, white balance, and manual control of red/green/blue (RGB) exposures.

³Windows-compatible personal computer. Most new PCs will have enough speed and RAM to handle MetaMorph. A large hard drive is recommended to accommodate the large microscope files.

4. Flush the liver/animal (Optional). Flush the liver with a 10-mL syringe filled with saline by inserting into the portal vein just below the liver. Follow with a 10-mL syringe filled with 10% neutral buffered formalin. This not only helps minimize possible ex vivo haptene formation but also helps retain the architecture of the organ. If other organs are also to be harvested, exsanguination, flushing, and fixing of the whole animal may be achieved by cardiac puncture and perfusion. A larger syringe (e.g., 50 mL) will be required.
5. Cut thin (2- to 4-mm thick) transverse sections of liver (or other organ of interest).
6. Place in tissue cassettes (labeled with pencil) and fix at 4°C for > 48 h.
7. Paraffin-embed tissue, section (5- to 6-µm thick), and mount on microscope slides (see Note 4).

3.3 Deparaffinization and Rehydration of Tissue Slides

At least one sample should be processed in duplicate in this procedure for primary antibody control (*see* Subheading 3.5., step 2.). Normally, the positive in vivo control (Subheading 3.1.) is selected for this control.

1. Slide racks for the Coplin jars have 10 slots (*see* Note 5). They may hold up to 20 slides if placed “back-to-back” (i.e., tissue side facing out). The jar should be filled with liquid to completely cover the slides (~225 mL). With a stir bar in the bottom of the jar, it is placed on stir plate (set to the lowest RPM). Incubate in each of the following solutions for 5 min:
 - a. Xylene (or alternate solution); repeat once
 - b. 100% ethanol
 - c. 95% ethanol
 - d. 70% ethanol
 - e. 30% ethanol
 - f. Nanopure water
2. Create a buffer dam around tissue (optional). While carefully avoiding drying of the section (do one slide at a time), paint a circle around the tissue section with the Dako pen (or nail polish), carefully avoiding touching the tissue with the solution (>3 mm away from the section). This buffer dam decreases the likelihood of the sample drying-out during antibody incubations. Place samples in a new Coplin jar containing fresh water.
3. Perform antigen demasking (optional). This procedure is strongly recommended for 3-nitrotyrosine immunohistochemistry.
 - a. Place slides in Coplin jar containing citric acid solution (Subheading 2.3., step 8.).

⁴Microscope sections must be of consistent high quality (e.g., few if any folds or tears). Should the laboratory lack a practiced hand at cutting and mounting tissues, it is highly recommended that the tissues be prepared and processed by a professional histopathology service.

⁵A number of newer and more rapid methods to deparaffinize and rehydrate microscope slides are available that use less reagent volumes than Coplin jars. However, it has been the experience of this author that deparaffinization/rehydration can be inconsistent and incomplete with these alternate approaches. These techniques are therefore not recommended for quantitative immunohistochemistry.

- b. Remove metal handle from slide rack. Place jar in a glass dish of water and microwave on high for 2.5 min (700-watt oven). Warning, buffer will get hot; avoid scalding.
 - c. Let cool for 3 min. Refill Coplin jar with citrate buffer, if needed.
 - d. Repeat steps b-c three times. Let cool in citrate buffer for 20 min.
 - e. Rinse sections in Coplin jar containing nanopure water for 5 min. Repeat once.
4. Rinse the samples in a Coplin jar containing PBS-Tween for 5 min. Repeat once.

3.4 Peroxidase and Protein Block of Tissue Sections

1. Make 250 mL of 3% hydrogen peroxide solution diluted in PBS-Tween (*see* Note 6). Pour into Coplin jar.
2. Incubate the slides for 5 min in peroxide solution.
3. Rinse the samples in a Coplin jar containing fresh PBS-Tween for 5 min. Repeat once.
4. Make PBS-Tween containing 1% BSA (PBA). You will need approx 250 μ L per slide.
5. Remove the slides from the Coplin jar one at a time. Pour off excess solution and pipette PBA into buffer dam (Subheading 3.3., step 2.) around tissue. Place on slide rack.
6. Incubate slides for 1 h at room temperature. Keep rack covered and hydrated by placing wet paper towels in the bottom of the rack. Add fresh solution as needed to avoid sample drying.
7. Proceed immediately to immunohistochemistry (Subheading 3.5.).

3.5 Immunohistochemical Detection of Haptene Signals

1. While samples are blocking (Subheading 3.4.), prepare the primary antibody solution by diluting the antibody in PBA (~250 μ L/slide). Historically, a 1:500 and 1:50 dilution of the 4OH-nonenal and 3-nitrotyrosine antibodies, respectively, were effective. Dilutions may vary on antibody batch.
2. Shake off excess PBA from slides one at a time and replace with primary antibody solution (*see* Note 7). The primary antibody control slide will receive PBA without antibody.
3. Incubate on slide rack (covered and hydrated) for 30 min (4OH-nonenal) or an hour (3-nitrotyrosine). Incubation times may need to be altered to improve signal to noise ratio. Rinse the samples in a Coplin jar containing fresh PBS-Tween for 5 min. Repeat once. Shake off excess PBS-Tween from slides one at a time and replace with secondary antibody solution. Place on slide rack and incubate for 15 min (if using the Dako EnVision kit). Dilutions and incubation times for secondary antibodies from other suppliers should be determined by manufacturer's suggestions.
4. Rinse the samples in a Coplin jar containing fresh PBS-Tween for 5 min. Repeat once.

⁶The Dako EnVision kit supplies a peroxidase block reagent. However, more consistent blocking has been achieved by our group using freshly diluted concentrated hydrogen peroxide in a Coplin jar.

⁷A critical component of quantitative immunohistochemistry is consistency, to avoid intra-assay variability. Careful attention should be paid so that the incubation steps for each sample are similar, especially in steps that require you to work with one sample at a time (e.g., Subheading 3.5., steps 2, 5, and 8). A simple solution is to measure the length of time required to add reagent to each sample.

5. Prepare DAB working solution according to manufacturer's instructions. Place a piece of white paper in the bottom of the slide rack. If the slide rack does not have a cover, make a cover with aluminum foil (DAB is light sensitive).
6. Apply DAB solution to each slide. Cover slides and incubate for 8-10 min. Use the in vivo positive control to determine the length of staining. When the brown DAB precipitate appears to not increase in this slide, stop the incubation. Transfer slides to a Coplin jar containing nanopure water.
7. Rinse Coplin jar by placing under a gentle tap of water for 6 min.
8. Transfer slide rack to a Coplin jar containing hematoxylin. Incubate in solution for 30 seconds while gently moving the slide rack up and down.
9. Return slide rack to Coplin jar containing water. Rinse under tap for 6 min.

3.6 Dehydration and Coverslipping of Slides

1. Incubate the slide rack in each of the following solutions for 3-5 min (see Subheading 3.3., step 1):
 - a. Nanopure water
 - b. 30% ethanol 100% ethanol
 - c. 70% ethanol 95% ethanol
 - d. 95% ethanol 70% ethanol
 - e. 100% ethanol
 - f. Xylene (or alternate solution); repeat once
2. Keep the slides in the second xylene solution (from Subheading 3.6., step 1f) until ready to be coverslipped. Place a thin bead of Permout on one long edge of a coverslip. Gently role the slide onto the coverslip. Capillary action will pull the pieces of glass together. Avoid bubbles over the tissue sections. Should bubbles occur wash off coverslip with xylene solution and recoverslip. Let the slides dry in the fume hood for at least 24 h.

3.7 Image Analysis of Immunohistochemistry

An absolutely critical point in performing quantitative image-analysis of immunohistochemistry is to ensure that all samples to be compared are analyzed simultaneously. It is recommended that before performing image analysis that the slides be critically analyzed under the microscope for any variation in chromogen signal not owing to the in vivo condition. Furthermore, image-analysis should be viewed as a means to quantitate differences between treatment groups, not to create. Specifically, the differences should be readily observable with the eye under the microscope.

For microheterogeneous tissues, such as the liver, a low power magnification (e.g., 100 ×, ocular and objective) is usually sufficient for analysis. For macroheterogeneous tissues (e.g., specific brain regions), a higher magnification may be required to focus on the area of interest. Should staining be located in a specific area within a tissue (for example, the perivenous area of the liver lobule), higher magnification photomicrographs may be also justified. It is recommended, however, that initial attempts be performed at lower magnifications, as this reduces the risk of investigator bias in acquiring the pictures. It is also a good idea to blind analysis of the tissues to reduce the risk of investigator bias.

1. Power-up microscope, camera and image-analysis computer. Turn-on the microscope light source to ~80% of full brightness and allow it to sit for at least 20 min. Most bright-field bulbs are unstable light emitters until they are sufficiently warmed-up. A simple way to validate that the bulb is stable is to take five consecutive photos of the same microscope region and validate that they are consistent in brightness, contrast and color.
2. While the system is warming-up, ensure that the microscope is optimized for taking pictures at the selected magnification (e.g., adjusting the diaphragm height and width). These techniques are routine to experienced microscopists.
3. Make sure that the image analysis software is set to manually determine exposure settings. Otherwise, the software may adjust the exposure between pictures, causing variations in staining color and intensity.
4. Using the image-analysis software, calibrate the exposure settings for the microscope field. First, move the microscope stage to a region of the slide that does not contain the tissue (but does have the coverslip) and defocus the image. Microscopes often have a gradient of light intensity across the field. Most image-analysis programs have a function to account for this gradient and normalize this gradient in captured photomicrographs (in MetaMorph, the command is “get flatfield”). Next, in the same area, determine optimal exposure settings for the digital camera (i.e., “white balance”). Fig. 1A,B compare the same microscope field before (A) and after (B) appropriate white balancing.
5. After performing flatfield and white balance commands, the photomicrographs captured by the software should be similar to those seen with eye. Validate this by capturing a representative image and comparing the digital picture with the image seen with the eye. If necessary, manually adjust the RGB exposure settings.
6. Enhance contrast with digital filtering (optional). Image analysis is much more sensitive to contrast than the human eye. Immunostaining may look very clear by manual analysis but may be fraught with noise during digital analysis. This noise can be alleviated by enhancing the digital contrast. For example, slightly increasing the blue exposure on the RGB setting will enhance the contrast between the DAB chromogen and the hematoxylin counterstain. However, care should be taken if this filtering approach is performed that artifactual signals are not introduced. Fig. 1C is the same microscope region as 1b, but after the blue exposure has been increased 10%.
7. Once steps 1 through 6 are completed, it is imperative that the neither the microscope nor the exposure settings are altered during the image capture phase. Select and capture 10 microscope fields from each section (assuming 10×10 magnification). The regions should be randomly selected with the caveat that staining artifacts (e.g., edges, folds, tears, etc.) should be avoided. Morphologic variations found in the tissue (e.g., very large vessels in the liver lobule) may also be avoided. However, due diligence should be paid to avoid investigator bias in selecting regions. Should a higher magnification be used to focus on a specific region of the tissue, it is recommended that the number of pictures captured be increased.
8. Repeat this step for all tissue sections to be analyzed.
9. Select an image file with strong positive staining for the haptene. Use this image to determine the color, intensity, and saturation ranges for the image-analysis (called “threshold” in MetaMorph). It is recommended that a “conservative” range be initially attempted to reduce noise in the sections. Fig. 1D shows the microscope region from

Fig. 1C determined by the computer to be within the threshold range for positive staining.

10. Pool the summary area data from each tissue section to determine the percentage of microscope field positive for the haptene signal. Use this value to determine group means.

3.8 In Vivo Modulation of Free Radical Production Using Genetically Modified Mice

The inherent reactivity of ROS/RNS makes it very difficult to directly measure them under in vivo conditions. Even electron spin resonance spectroscopic analysis of free radicals usually requires a spin-trap to stabilize the radical signal long enough for detection. Therefore, although assessment of changes in ROS/RNS by the techniques described above are useful, these 'footprints' do not give any information on the type(s) and source(s) of reactive species produced that caused these changes.

To date, most work has employed chemical inhibitors of suspected prooxidant enzymes. The effect of these drugs in vivo may not necessarily be specific to inhibition of the enzyme in question. Therefore, caution should be used when basing conclusions on work with pharmacologic inhibitors. One advance towards this end in the alcohol field has been the ability to address key questions at the molecular level by using genetically-altered mice (e.g., knockout/transgenic (13)). Such strategies have their own potential pitfalls, such as induction of compensatory mechanisms in knockout mice, which may cause false-positive or -negative results. (*see* Note 8 for general guidelines on the use of genetically altered mice.)

References

1. Koppenol WH. The centennial of the Fenton reaction. *Free Radic. Biol. Med* 1993;15:645–651. [PubMed: 8138191]
2. Gerschman R, Gilbert DL, Nye SW, Dwyer P, Fenn WO. Oxygen poisoning and X-irradiation: a mechanism in common. *Science* 1956;119:623–626. [PubMed: 13156638]
3. Harman D. Aging: A theory based on free radical and radiation chemistry. *J. Gerontol* 1956;11:298–300. [PubMed: 13332224]
4. McCord JM, Fridovich I. Superoxide dismutase: An enzymatic function of erythrocuprein (hemocuprein). *J. Biol. Chem* 1969;244:6049–6055. [PubMed: 5389100]
5. Sies, H. Oxidative stress: introductory remarks, in *Oxidative Stress*. Sies, H., editor. Academic Press; London: 1985. p. 1-8.
6. Di Luzio NR. A mechanism of the acute ethanol-induced fatty liver and the modification of liver injury by antioxidants. *Lab. Invest* 1966;15:50–63.

⁸The following are general guidelines for using genetically altered mice in experiments. These guidelines are derived from the Banbury conference on genetic background in mice (14), and have been adopted by many scientific journals:

- a. Genetic background of mouse strain must be clearly reported.
- b. Common strains of mice should be used to aid reproducibility by other laboratories.
- c. The genetically modified strain should be backcrossed into a common background strain.
- d. The strain should be backcrossed into the wild-type strain at least four times to avoid genetic variation at other sites besides the locus of interest.
- e. Genetically modified line should not be maintained by crossing homozygotes, but rather maintained by backcrossing heterozygotes against the wildtype strain. This avoids the potential of genetic drift of the modified strain away from the background strain.

Results with modified strain should be validated with multiple generations. Further, weight-of-evidence can be increased by comparing pharmacologic targeting of the same protein with the knockout strain.

7. Shaw S, Jayatilleke E, Ross WA, Gordon ER, Lieber S. Ethanol-induced lipid peroxidation: Potentiation by long-term alcohol feeding and attenuation by methionine. *J. Lab. Clin. Med* 1981;98:417–424. [PubMed: 7264437]
8. Tomasi A, Albano E, Biasi F, Slater TF, Vannini V, Dianzani MU. Activation of chloroform and related trihalomethanes to free radical intermediates in isolated hepatocytes and in the rat *in vivo* as detected by the ESR-spin trapping technique. *Chem. Biol. Interact* 1985;55:303–316. [PubMed: 3000632]
9. Reinke LA, Lai EK, DuBose CM, McCay PB. Reactive free radical generation in the heart and liver of ethanol-fed rats: Correlation with *in vitro* radical formation. *Proc. Natl. Acad. Sci. USA* 1987;84:9223–9227. [PubMed: 2827172]
10. Rashba-Step J, Turro NJ, Cederbaum AI. Increased NADPH- and NADH-dependent production of superoxide and hydroxyl radical by microsomes after chronic ethanol treatment. *Arch. Biochem. Biophys* 1993;300:401–408. [PubMed: 8380969]
11. Knecht KT, Bradford BU, Mason RP, Thurman RG. *In vivo* formation of a free radical metabolite of ethanol. *Mol. Pharmacol* 1990;38:26–30. [PubMed: 2370854]
12. McKim SE, Gabele E, Isayama F, Lambert JC, Tucker LM, Wheeler MD, Connor HD, Mason RP, Doll MA, Hein DW, et al. Inducible nitric oxide synthase is required in alcohol-induced liver injury: studies with knockout mice. *Gastroenterology* 2003;125:1834–1844. [PubMed: 14724835]
13. Arteel GE. Oxidants and antioxidants in alcohol-induced liver disease. *Gastroenterology* 2003;124:778–790. [PubMed: 12612915]
14. Banbury Conference on genetic background in mice. Mutant mice and neuroscience: recommendations concerning genetic background. *Neuron* 1997;19:755–759. [PubMed: 9354323]

Fig. 1. Effect of LPS on 4OH-nonenal adduct accumulation in mouse liver as determined by quantitative immunohistochemistry. Representative photomicrographs are shown depicting 4OH-nonenal immunostaining of a liver section (100×) of a mouse 24 h after injection with LPS (10 mg/kg intraperitoneally). The same microscope region is shown before (**A**) and after (**B**) white balancing of the image-analysis software. Note that the acellular space in (**A**) is light yellow instead of white. (**C**) is the same as (**B**), but after the blue exposure is increased 10%. Note that the contrast between the DAB chromogen (brown) and the hematoxylin counterstain (blue) is enhanced by digitally increasing the blue exposure. (**D**) shows the microscope region from (**C**), determined by the computer to be within the threshold range for positive staining