METHODS USED IN INTERNAL MEDICINE IMAGING CORE

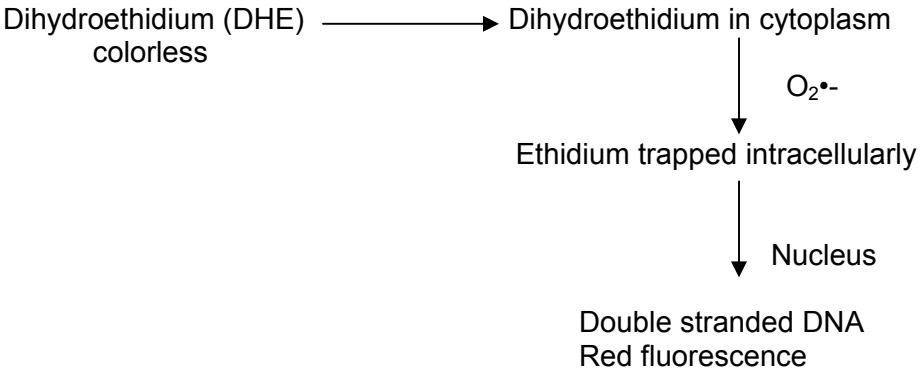
1. Detection of ROS ($O_2^{\bullet-}$ and H_2O_2) in vascular tissues

One central theme unifying the projects outlined in this grant is that inflammation within tissues of the cardiovascular system leads to increased production of ROS, which in turn ultimately leads to vascular dysfunction and hypertension. Therefore it is important to localize pro-inflammatory markers or cells within different areas of cardiovascular tissue and identify those areas associated with increased ROS production.

1.1. Detection of $O_2^{\bullet-}$

Recently, a cytochemical method for detection of $O_2^{\bullet-}$ using the oxidative fluorescent dye dihydroethidium (DHE) (Invitrogen) and laser scanning confocal microscopy (LSCM) has been developed for localization of $O_2^{\bullet-}$ levels in situ in endothelial and vascular smooth muscle cells and in enzymatically intact 30 μm-thick vascular sections (36). Optical sectioning with a confocal microscope eliminates the out-of-focus glare characteristic of conventional fluorescence microscopy of thick specimens and provides for high resolution, high contrast imaging of the tissue sections. Detection of ethidium within tissue sections, which maintains the architectural structure of the vascular wall, can be combined with indirect immunofluorescence labeling with specific cell type markers for endothelial cells, smooth muscle cells, cardiac myocytes, fibroblasts and/or lymphocytes/macrophages to delineate regional and cellular differences in levels of ROS under a variety of stimuli and conditions (37). These conditions include production of ROS either in response to physiological stimuli or to pathological conditions such as atherosclerosis or hypertension.

Projects 2 and 4 will be assessing $O_2^{\bullet-}$ by ethidium staining in enzymatically intact 30 μm-thick fresh frozen aortic sections. Nonfluorescent DHE, which is permeable to cells, is oxidized by intracellular $O_2^{\bullet-}$ to ethidium, which intercalates into the DNA in the nucleus and fluoresces red (Excitation/Emission maxima=510/595 nm). This punctate nuclear staining is easily distinguished from nonspecific autofluorescence of elastic layers. **The ethidium fluorescence/confocal microscopy combination is currently the most accepted measure of qualitative intracellular in situ $O_2^{\bullet-}$ production within tissue.**



1.2. Preparation of tissues for $O_2^{\bullet-}$ detection

For ethidium fluorescence, tissues will be rinsed immediately in cold (4°C) phosphate buffered saline (PBS), placed in Tissue-Tek optimal cutting temperature compound (OCT, Miles Laboratories), and snap frozen in liquid nitrogen. Within 2-4 hours after harvest, 30 μm-thick cryosections of unfixed frozen segments will be obtained using a Leica CM3050 or a Microm HM505E cryostat and placed on a glass slide. DHE will be suspended in dimethyl sulfoxide (DMSO) at stock concentrations of 10 mM, stored in frozen aliquots at -80°C until use, and diluted for a final concentration of 10 μM in PBS. DHE will be topically applied to each tissue section, which is then mounted with a coverslip. Slides will be incubated in a light-protected humidified incubator at 37°C for 30 minutes. Specificity of staining will be confirmed by ablation of the signal by preincubation of the sections with 500 U/ml PEG-superoxide dismutase (SOD). Some sections stained with

DHE will be counterstained with either Hoechst 33258 (Ex:Em=340/450 nm) or 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Ex:Em=345/458 nm), DNA binding fluorophores that label all nuclei, or with cell-specific antibodies as described below. Sections will be examined within 8 hours of harvest with confocal microscopy as described below. The number of ethidium- and Hoechst/DAPI-positive cells within 5 representative optical sections will be analyzed with Image-Pro Plus, and $O_2^{\bullet-}$-producing cells will be expressed as a percentage of total cells. Semi-quantitative data on ethidium fluorescence (indicating $O_2^{\bullet-}$ production) will be analyzed on fluorescence confocal images as described below.

1.3. Detection of H_2O_2

To evaluate the presence of peroxides in frozen sections, the peroxide-sensitive dye chloromethyl-2',7'-dichlorofluorescein diacetate (DCF-DA: Invitrogen: 10 μM) and its oxidant-insensitive analogue carboxyl-DCFDA (Acros) will be used in place of DHE in some sections. CM-H_2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases, and fluoresces green (Ex:Em=502/523 nm) when oxidized by H_2O_2. Specificity will be confirmed by preincubation with PEG-catalase (350 U/ml). Visualization by confocal microscopy will be performed as described below. Because the autofluorescence of elastin (Em: > 515 nm) (38) of the internal elastic laminae in transverse sections of vessels overlaps with the emission of DCF-DA (523 nm), spectral imaging and linear unmixing using the Zeiss LSM 510 META software algorithms will be used to separate the spectral profiles of elastin from those of DCF-DA in the vascular cells (39), particularly the smooth muscle cells within the elastin layers.

1.4. Confocal imaging of sections for ROS detection

After DHE staining, sections will be examined with a Zeiss LSM 510 META equipped with a blue diode (405 nm emission line), an argon ion (458/477/488/514 nm emission lines), a green helium/neon (HeNe) (543 nm emission line), a red HeNe (633 emission line) lasers and a polychromatic detector for spectral imaging (META). For orientation of vascular cell layers, autofluorescence of elastin in the internal elastic lamina will be visualized by excitation of tissue sections with the 488 nm line combined with a 505-530 nm band pass filter. Fluorescence of ethidium (Ex:Em=510/595 nm) will be detected with excitation line 488 nm and emission lines 580-646 nm in the META detector. Tissue sections from control or animal models will be processed and images acquired in parallel with identical laser power, pinhole, gain and offset settings. For semi-quantitative data on ethidium fluorescence (indicating $O_2^{\bullet-}$ production), fluorescence confocal images will be acquired in parallel as 12 bit (4096 intensity levels) and subsequently analyzed with Image-Pro Plus software. Relative fluorescent units (RFU) will be reported as mean ± standard error. Data will be analyzed with an unpaired two-tailed *t*-test or a one way ANOVA with a post-hoc Bonferroni's comparison. Statistical significance will be accepted if the null hypothesis is rejected at $p \leq 0.05$. For sequential excitation of Hoechst 33258 and ethidium, laser emission lines of 405 and 488 nm will be used for excitation, with emission filters of LP 420 and 580-646 nm (with the META detector), respectively. Images will be analyzed by Image-Pro Plus software and exported into Adobe PhotoShop 7.0.1 for image presentation.

The feasibility of using DHE as a marker for $O_2^{\bullet-}$ production has been previously demonstrated (36). Results from images taken in our Microscopy and Histology Core revealed an increase in $O_2^{\bullet-}$ production in the arterial wall as detected by ethidium fluorescence in both human disease (Figure 1) (17) and in an animal model of hypertension (Figure 2).

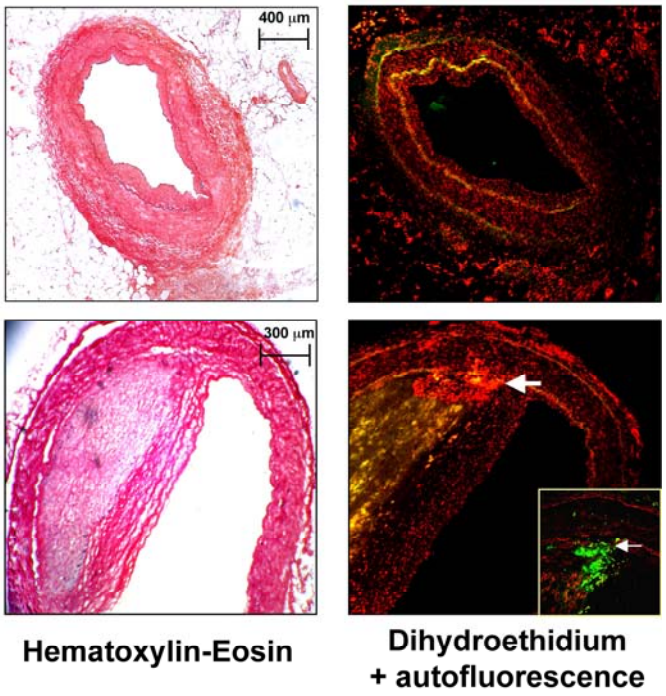


Figure 1. Alternate transverse sections (H+E staining in left panels and ethidium fluorescence in right panels) of nonatherosclerotic (top) and atherosclerotic (bottom) human coronary arteries showing intense ethidium (increased $O_2^{\bullet}$ production) at the shoulder of the plaque (arrow) and in the intima. Autofluorescence of the elastic laminae and the acellular, cholesterol-rich core are shown in yellow. Inset: Macrophage staining (green) of shoulder region with CD68. From Sorescu et al. (17).

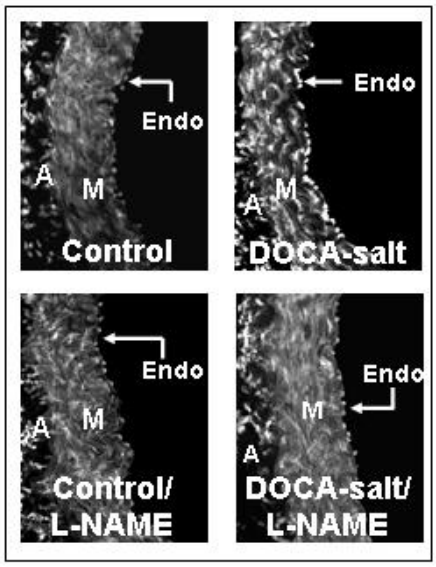


Figure 2. In situ detection of $O_2^{\bullet}$ production in 30 μm-thick sections of aortas from sham-operated and DOCA-salt hypertensive mice using DHE staining. The increase in DHE staining in the endothelium of DOCA-salt-treated animals is eliminated by the NOS inhibitor L-NAME. Data are representative of 4 separate experiments. Endo=Endothelium; M=Media; A=Adventitia. Micrograph courtesy of U. Landmesser.