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In vivo capillary diameters in the stria vascularis and spiral ligament of the guinea pig cochlea

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Blood microvessels in the membranous lateral wall of the cochlea were examined using intravital microscopic techniques. A video analysis system made serial diameter measurements at 1 μm intervals along the length of selected vessel segments during four experimental conditions. For each vessel segment, the serial measurements were statistically converted into a single diameter estimate, such that the flow resistance in a uniform vessel of this diameter would equal the resistance of the real non-uniform vessel.

Nominal vessel diameters found (spiral ligament: 9–12 μm; stria vascularis: 12–16 μm) were nearly double those reported earlier in histological observations (Axelsson, 1968). During stimulation the largest diameter change seen was a 3.7% dilation (about 0.5 μm) in response to breathing 5% CO_2 in oxygen. Theoretically, this change could reduce vascular fluid resistance by 16%, nearly enough to explain the observed flow increase of 20%. No diameter changes occurred for 5% CO_2 in air despite a 50% flow increase, nor for air pressure pulses applied at the tympanic membrane. Round window electrical stimulation of 50 μA also produced dilation ($< 2.5\%$), but higher current levels were ineffective. In general, blood flow increases seen in this study could not adequately be attributed to the small lateral wall vessel diameter increases nor systemic causes, suggesting that lateral wall blood flow in these instances is dependent on control within the modiolus.

Cochlear blood flow; Capillary diameter; Carbon dioxide; Electrical stimulation; Image analysis; Intravital microscopy

Introduction

Evidence that insufficient cochlear blood flow may contribute to the etiology of a wide variety of auditory disorders comes from indirect (e.g., Axelsson and Vertes, 1982; Maass et al., 1978) and direct (e.g., Hultcrantz et al., 1980; Dengerink et al., 1984) studies of inner ear blood flow. However, there is considerable controversy raised by the seemingly conflicting results (see for example the discussion of sound-induced oxygen change in the inner ear: Nuttall et al., 1981; Thorne and Nuttall, 1988).

In the attempt to understand the mechanisms underlying these complex results, some studies have examined the role of vascular vessel diameters in cochlear blood flow control. The most frequently employed method of measuring cochlear vessel diameter has been a histological examination, often shortly following some noxious stimulus. This method allows high (optical) resolution, post-mortem measurements of many of the vascular beds within the cochlea. However, the time course of vascular phenomena is difficult to follow, and the accuracy of the post-mortem measurements is open to question since little is known about what happens to the vessels during fixation (Axelsson, 1968; Sobin and Rosenquist, 1973).

Alternatively, in vivo methods can be used to examine some of the vascular beds within the cochlea, if care is made to ensure the normality of the tissues. In vivo methods also permit high accuracy measurements, and short-term events can be followed quite readily.

In order to study the control of blood flow in the cochlea, the current investigation used three broad classes of stimuli for the characterization of

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the responsiveness of lateral wall cochlear microvessels. The vessels were measured from video images recorded using an intravital microscope and epifluorescence techniques. The vessel diameters were measured automatically using an image analysis algorithm (Miles, 1987), which improved accuracy and eliminated observer bias. Each selected vessel was measured at 1 μm increments along chosen segments of its length, avoiding the implicit assumption that each vessel was of uniform diameter. A means of converting these serial measurements into a single physiologically meaningful parameter was developed. The results show that living vessels are considerably greater in diameter than stated in some previous studies of fixed tissues; and that significant diameter changes can occur in the lateral wall vessels for some stimuli.

Methods

Seventeen pigmented guinea pigs of both sexes (240–460 g) demonstrating robust Preyer's reflex, were used. After tranquilizing with diazepam (5 mg/kg ip), analgesia was provided by fentanyl (0.32 mg/kg im). Anesthesia was maintained by a supplementary half-dose of fentanyl every 30 min and a half-dose of diazepam every 2 h. The diazepam-fentanyl combination was chosen based on its ability to maintain blood pressure at approximately 70 mmHg which is a slightly elevated blood pressure over the unusually low values observed for guinea pigs (approx. 53 mmHg) (Brown et al., 1988). A ventilation tube was inserted into the trachea, with the animals breathing freely through it for most of the duration of the experiments. Artificial respiration was used during electrical stimulation when the animals were paralyzed with curare (0.3 mg im).

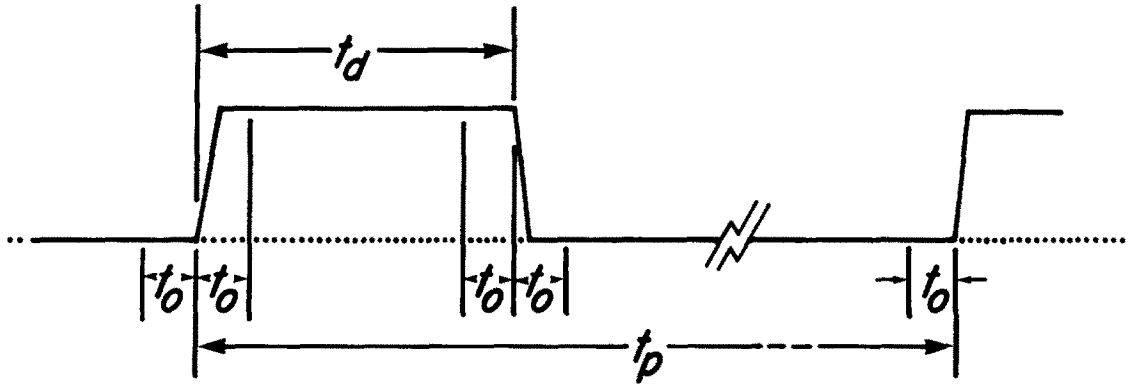
The left cochlea was exposed by a ventro-lateral dissection of the neck and opening of the bulla. The tympanic membrane and middle ear ossicular chain remained intact. Most of the observations were made through a small opening in the external bony wall of the cochlea adjacent to the stria vascularis of the first or third turn (Nuttall, 1987). Visualizing the vessels within the thick tissue was greatly aided by a fluorescein-labeled dextran solution (50 mg of 150 kDa FITC-dextran in 1 ml physiological saline) which was slowly injected into the cannulated jugular vein of the animal in partial doses over the course of the experiment. The use of the dye ensured that the measurements would be of the entire lumen of the vessels and would not include the vessel walls.

Intravital microscope components were held by a positioning carriage, allowing precise movement control in all three axes, and mounted on a vibration-isolated table. The tissues were illuminated with a 75 watt xenon lamp coupled to the vertical illuminator through a liquid light guide. A set of optical filters suppressed all light going to the observer except that near the fluorescence frequency. The low fluorescence intensity made it essential that a highly sensitive camera be used to capture the images. For the current experiments, a silicon-intensified-target (SIT) video camera was used (Dage MTI-66, Michigan City, IN). Its response to light intensity change (gamma) was modified to be nearly linear.

Rectal temperature of the animal was maintained at $38 \pm 1°$ C with a servo-regulated heating blanket, and head temperature was independently maintained by a heated head-holder. Temperature of the surgically exposed cochlea was maintained by supplemental heating with a heat lamp. The cochlea was bathed with heated, humidified, 5% CO_2 in nitrogen mixture to avoid any possibility of supplementing tissue gas exchange through the openings in the cochlear wall. Heart rate was measured with stainless-steel needle electrodes under the skin of the thorax. Mean blood pressure was measured in the cannulated left common carotid artery using a pressure transducer (Gould P23, Gould, Inc., Oxnard, CA). Data from animals with mean blood pressures dropping below 55 mmHg were excluded from the final analysis (electrically stimulated animals were treated differently; see below). Respired gas concentrations (O_2 and CO_2) from the tracheotomy tube were continuously sampled and measured by gas analyzers.

Stimulus conditions

Four different stimulus types were used (Fig. 1). The first stimulus set consisted of two negative and two positive 2 s trapezoidal pressure pulses applied via a speculum to the external auditory meatus. This stimulus type was chosen based on preliminary observations of marked blood flow



Stimulus type	Acquisition offset t_o	Stimulus duration t_d	Inter-stimulus period t_p	Stimulus sequence
Pressure	0.25 s	2.0 s	7.0 s	−0.35, +0.35, −0.88, +0.88 [mmHg]
CO_2 in O_2 CO_2 in air	15 s	3 min	15 min	randomized order
Electrical	15 s	60 s	3 min	0.05, 0.10, 0.20, 0.50, 1.0, 2.0 [mA, 0 to peak]

Fig. 1. Stimulus types and timing. Four kinds of stimuli were present with predetermined time delays between measurement (image acquisition), and stimulus onset or offset. As permitted by the condition of the animal (in 12 out of the 17 cases), all four kinds of stimuli were used in each experiment. Trapezoidal pressure pulses were applied first, and the electrical stimulus of the round window last.

reduction in cochlear basal-turn collecting venules during normal middle ear muscle contractions. The maximum pressure increase (approx. 120 Pa) caused the handle of the malleus to travel about the same distance as a strong contraction of the tensor tympani muscle, which was cut in these experiments to eliminate any spurious contractions. Five seconds were allowed between stimuli for recovery. Some observations of collecting venules, in the first or third turn of the cochlea, were done through a section of thinned bone before any opening was made in the cochlea.

Two different CO_2 stimuli were given in random order: 5% CO_2 in air, and 5% CO_2 in oxygen. The animals freely respired the selected gas for 3 min, followed by at least 12 min of freely respired air. This stimulus is of interest since CO_2 mixtures have been used clinically for patients with hearing disorders such as sudden deafness (e.g., Giger, 1979).

The last stimulus set consisted of electrical stimulation of the cochlea. A 1 kHz sinusoidal current [50 μA to 2 mA (zero to peak), 6 amplitudes in a 5–10–20 ascending sequence] was supplied by an electrically-isolated stimulator to the round window through a platinum-iridium ball or wire-loop electrode. The return electrode was a silver-silver chloride electrode in the neck tissues or a platinum-iridium wire in a hole in the apex of the cochlea. Each current level was applied for 60 s, followed by 2 min of recovery. To prevent the possibility of animal movement from the current, the animals were curarized and artificially respirated during the current stimulus set. Measurements of respiratory CO_2 gas concentration were used to adjust the respiratory volume. The artificial respiration often resulted in a reduction in systemic blood pressure to levels below normal awake resting values (Brown et al., 1988) because of positive intrathoracic pressure during artificial

(pump) respiration. The 55 mmHg criterion was relaxed during these electrical-stimulation experiments which required artificial respiration. Electrical stimulation of the cochlea was used because of its potential for causing local vascular changes. Experiments using the laser Doppler flowmeter (Sillman et al., 1987) have shown increased blood flow during round-window, electrical stimulation.

Image analysis

The camera output was continuously displayed during each experiment on a video monitor and stored on a video recorder (Sony VO-5800). Blood flow rate was measured by counting the number of frames (at 60 per s) for a relatively well defined object within a vessel to travel a known distance. For diameter measurements, each complete experiment was reduced to a minimum of 52 digitized, averaged images, each representing a different time instance of a vascular bed for each cochlea. An image was taken shortly before and after stimulus onset, shortly before and after offset, and again after a predetermined recovery period. Each image was formed by averaging four separate video frames from the recorded video signal. For the pressure experiment, the four frames were taken by sampling every other recorded frame (approx. 0.25 s total interval). For the other experiments, the four frames were taken at 1 s intervals (4 s total interval). For a few images, it was necessary to substitute a different nearby frame for the ordinarily selected one, to avoid averaging a frame slightly displaced by small head motion. Such motion was generally related to the respiratory cycle. The spatial resolution was limited by the camera, resulting in images of 240 $\times$ 236 pixels. The resulting images were filtered, the uneven background illumination from out-of-plane vessels was corrected, and the pixels were slightly stretched vertically to correct the aspect ratio. The resulting pixels measured 1.06 μm on each side.

The most important step in the measurement of microvessel diameters was determining the exact path of the vessel edges in each two-dimensional image. A combination of several methodologies was used to yield accurate diameter measurements, complete with error estimates, without undue loss of computational efficiency (Miles, 1987; Miles and Nuttall, unpublished data).

The central feature of this method is a vessel model based on the physics of fluorescence and the assumed circular shape of the vessel cross section. The close match between the mathematical model and recorded intensity profiles of real vessels was demonstrated (Fig. 2A, B). Diameter measurements were interpolated from the matching of the profile of the fluorescence and a set of the model profiles. This provided sub-pixel resolution of the diameter estimates.

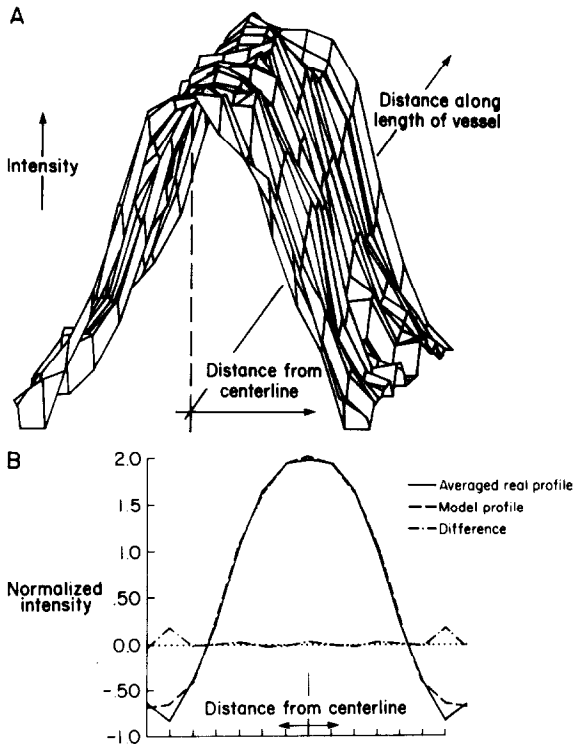


Fig. 2. A. Series of real vessel intensity profiles. A series of 21 intensity profiles measured from a length of a straight, uniform-diameter vessel segment. These were taken during an in vivo examination of vascular patterns of a guinea pig cochlea, following the intravenous injection of an FITC-dextran solution. B. Comparison of model and average measured intensity profiles. The averaged real-vessel profile was obtained by averaging the intensity longitudinally along the vessel (from A). The theoretical (model) profile was selected from a family of functions with only two parameters: vessel diameter and optical resolution blur. The profiles were normalized by subtracting the mean intensity, and dividing by the average signal power. The mismatched outside of the vessel boundaries is not significant.

While earlier studies have made, at most, a few diameter measurements along each vessel, the image analysis algorithm of this study made measurements at 1 μm steps along each vessel-segment's length. Because of the enormous amount of data subsequently generated, some means is required for reducing the number of individual measurements, without losing the effect that each piecewise diameter would have on blood flow. The method developed collapses a large number of diameter measurements into a single 'equivalent diameter', which is the diameter of a uniform vessel having the same length and the same resistance to blood flow as a vessel with non-uniform diameter (Miles and Nuttall, unpublished data).

Results

The normal patterns of blood flow did not seem to change significantly during most experiments. Blood flow in the lateral wall was typically of constant velocity. Occasionally in these experiments, the flow of blood in a single vessel segment would completely stop. Before complete stoppage, the flow typically would become more variable, i.e., with occasional brief halts. Once flow in a vessel had halted for more than a minute, it was rare that it would start again, regardless of any experimental stimuli or the rate of flow in adjoining or near-by vessels. In no case was any constriction in the lumen observed which might have caused the flow to stop. These stoppages appeared to be a result of damage during the surgical preparation. No data were used from vessels in which blood later ceased to flow. If blood flow stopped in more than a few vessels during the experiment, all of the data from that animal were excluded. No sphincter-like phenomena were visually apparent in any of these experiments.

About 100 000 computer-derived individual diameter measurements were made of 100 vessel segments in 17 animals. An analysis of the individual diameter measurements made by the computer showed that these were too variable to reliably show localized constrictions in a vessel for a single stimulus presentation. This variability was mostly due to camera noise and to optical distortions caused by observing through the lateral wall tissues. The results subsequently reported are of averages of normalized equivalent diameters which were obtained by dividing each diameter by the mean equivalent diameters, taken before stimulus presentation.

The nominal equivalent diameters observed in the lateral wall are shown in Fig. 3. Since the cochleae were not histologically examined after each experiment, it is not possible to be completely certain of the classification of these vessels. Furthermore, in this study no attempt was made to uniformly or randomly sample the diameters of each possible type of vascular bed. Our judgment, however, is that the equivalent diameter of the spiral ligament vessels is on the order of 9–12 μm, and of the stria vascularis vessels is on the order of 12–16 μm. The largest vessels measured were collecting venules. The smallest vessels seen were suprastrial vessels. These were infrequently observed, and were about 7 μm in size.

The pressure stimulus elicited no measurable changes in any of the monitored, physiological indicators. The stimuli were too brief to measure blood flow rate changes. No significant diameter changes were detected, either with or without the bony shell in place. The mean, absolute, measured, diameter change was less than 0.6%, equivalent to a variation in calculated vascular resistance of less than 2.5%. The constancy of the diameter measurements for this stimulus set demonstrates the stability of the measurement system.

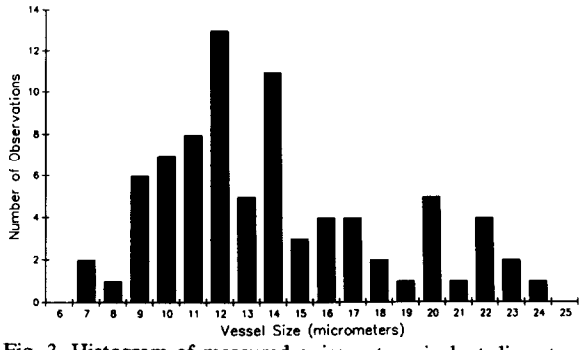


Fig. 3. Histogram of measured quiescent equivalent diameters (in μm). This figure shows the distribution of the resting, equivalent diameters measured during all of the experiments. The spiral-ligament vessels were 9–12 μm, and the stria-vascularis vessels were 12–16 μm. Some collecting venules also appear here as the largest vessels.

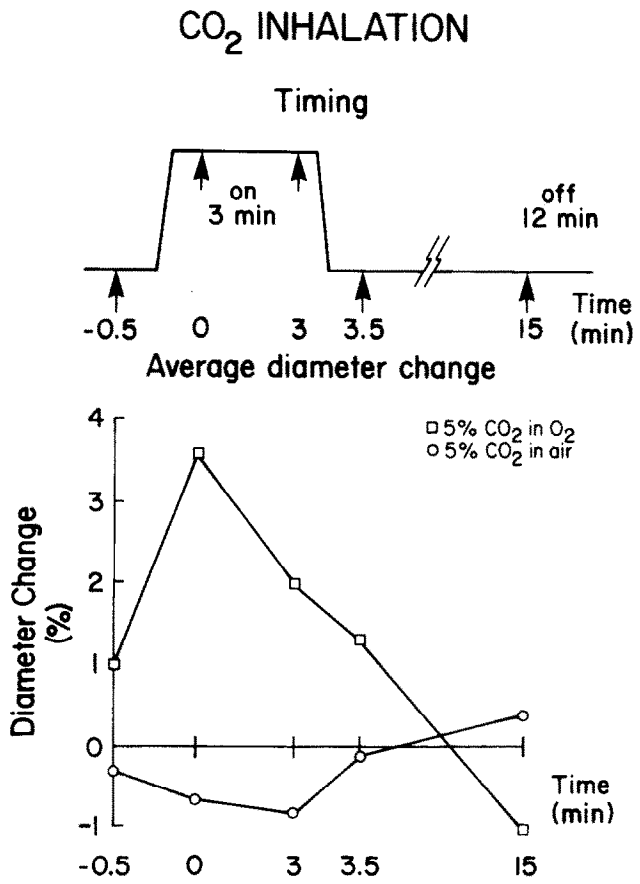


Fig. 4. Diameter change while breathing 5% carbon dioxide mixtures. This graph shows the strong dependence of dilation on the time of breathing gases containing CO_2 (timing given by upper graph). The diameter increase with the CO_2 in oxygen mixture was significant ($P > 0.999$), but the CO_2 in air mixture was not significant even at the $P > 0.75$ level. Paradoxically, very little dilation occurred for the CO_2 in air mixture. Heart rate and systemic blood pressure were essentially identical for these two stimuli, averaging 355 beats/min (±20) and 70 mm Hg (±5). Blood pressure increased 10% when breathing either CO_2 mixture.

The two CO_2 mixtures both produced a mean increase in blood pressure of 10% and mean decrease in heart rate of 1.5%. Blood flow velocity increased about 20% for the oxygen mixture and 50% for the air mixture. Hyperpnea was particularly evident during the air mixture administration. The largest diameter change seen for any of the tested stimuli occurred for the CO_2 in oxygen stimulus in which the mean diameter increased by 3.7%. This small change in equivalent diameter was not evident in watching the video monitor. Paradoxically, vessel dilation was present for the oxygen mixture but not for the air mixture, as can be seen graphically (Fig. 4) and through statistical tests. The effect was independent of the order in which the two CO_2 mixtures were presented. Vessel diameter increase was significant to $P > 0.999$ by the nonparametric Kruskal-Wallis k-sample location test (a multi-dimensional extension of the well-known Mann-Whitney U-test; Hollander and Wolfe, 1973). For most vessels, 3.7% represents an increase of about 0.5 μm. The CO_2/air-mixture dilation was not significant as low as $P > 0.75$.

The form of the distribution of the vessel sizes was altered by the CO_2 in oxygen stimulus ($P > 0.99$) by the two-sided Kolmogorov-Smirnov k-sample distribution comparison test (Conover, 1971). This was further explored by grouping vessels by turn and by size (Fig. 5A). The response of

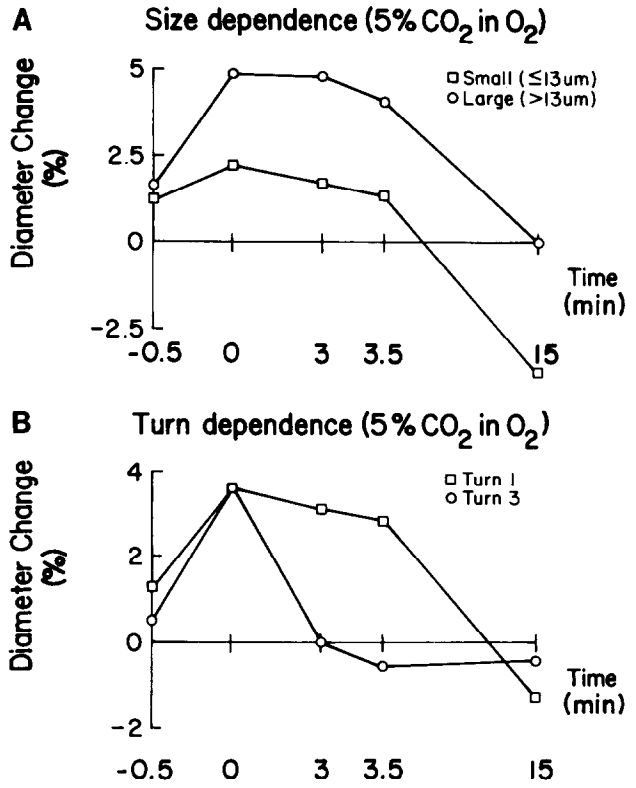


Fig. 5. A. Relative dilation depending on vessel size. This graph shows the difference in dilation between small vessels (<13 μm) and large vessels (>13 μm) with free respiration of CO_2 in oxygen. While the larger vessels appear to have a greater response to the stimulus, this was significant only at the $P > 0.75$ level. Closer examination of the data showed a large variability in the response relative to the number of sample vessels in this group. Timing of breathing the CO_2 is the same as for Fig. 3. B. Variation of dilation depending on location. This graph shows the difference in dilation between first turn and third turn with free respiration of CO_2 in oxygen. The dilation in the first turn is significantly stronger and more prolonged than that in the third turn ($P > 0.95$).

the vessels of the first turn was greater and more prolonged ($P > 0.95$) than those of the third turn in which vessels started to recover before the stimulus was terminated (Fig. 5B). No significant differences in response were seen between the larger and smaller vessels. Recovery from the stimuli appeared complete at the end of the allotted time, judging from gross, physiological conditions and from vascular diameter measurements.

The round-window, electrical stimulation resulted in nearly doubling the blood pressure at the highest current level despite essentially constant heart rates, curarization, and placement of the return electrode in the apex of the cochlea. Blood flow velocity increased up to three times the initial rate for the largest stimulus levels. Vessel diameters were generally constant, although a significant dilation occurred ($< 2.5\%$) for the first two (smallest) stimuli of 50 μA and 100 μA. The effect of the last two (largest) electrical stimuli persisted such that the recovery might not have been completed when measurements were taken. As for the CO_2 in oxygen stimulus, the dilation was greater in the first turn than in the third. Differences in dilation with respect to vessel size (independent of vessel type) may exist but were not statistically significant with the present data.

Discussion

A number of methods were used to verify the accuracy of the semi-automatic computer algorithm used for measuring vessel diameters. Human observers making diameter measurements of

fluorescent objects are highly sensitive to video display brightness and contrast, and typically underestimate the true diameter (Spears et al., 1983). Physical models of microvessels which implement the full range of vessel and imaging conditions at these dimensions are difficult to construct. As a result, computer-generated (synthetic) images of blood vessels incorporating a wide variety of correlated and uncorrelated noise were used to calibrate the system.

Despite some small uncertainty in identifying the specific types of lateral-wall vessels, there is no doubt that the vessels measured in these experiments are much larger than those reported in an extensive, histological study (Axelsson, 1968). It has been suggested that diameter values made using post-mortem techniques are subject to fixation errors (Sobin and Rosenquist, 1973; Axelsson, 1968). In a more recent post-mortem study (Dengerink et al., 1987), vessel diameters were measured in terms of red blood cell (RBC) diameters. Assuming that the RBC diameters were not subject to gross shrinkage, post-mortem diameter measurements in this later report were at least as large as the current in vivo measurements. The diameters of each vessel class reported here are also close to those reported by Nuttall (1987), using a human observer to make the measurements. The accuracy is affected, of course, by the correct classification of each vessel type. Although histological verification (not carried out in this study) is the ultimate standard, knowledge of the location of the fenestra and experience with the general pattern of the vascular network allows one to have confidence that 1) arterioles and venules are largely excluded and 2) the strial capillaries and spiral-ligament capillaries form a bimodal population. Given the apparent agreement of the most recent post-mortem results (Dengerink et al., 1987), and in vivo measurements obtained without the aid of the computer algorithm (Nuttall, 1987), the current measurements provide accurate estimates of normal vessel sizes.

One implication of these diameter measurements is to suggest that studies of guinea pig cochlear blood flow using microsphere techniques should use larger microspheres than have been used in the past (e.g., Hultcrantz et al., 1980; Prazma et al., 1983). However, the trapping of small (9–10 μm) microspheres may still occur even though equivalent vessel diameters are greater, since the vessels are not uniform circular cylinders. Currently, the efficiency of small microsphere trapping is not known and may be dependent on factors which cause minor variations in vessel diameter.

Quiescent flow rates measured in the current study agreed closely with previous measurements by Perlman and Kimura (1955) and Nuttall (1987). Increases in flow rates with 5% CO_2 breathing are consistent with changes in intracochlear oxygen tension observed for the same gas concentration (Murata and Fisch, 1977; Prazma, 1982). Whole cochlear blood flow increases, measured using the microsphere technique (Hultcrantz et al., 1980), were maximal only for breathing 7% CO_2 in air. Small and variable responses have been measured with the laser Doppler flowmeter for breathing 5% CO_2 in air (Sillman et al., 1987).

No diameter changes were visible to the observer for any of the stimuli. The small dilations were revealed through image analysis and by grouping of large numbers of measurements. Short term pressure changes had no measurable effect, whether applied externally, via the middle ear ossicles, or internally, as a result of increased blood pressure during the maximum electrical stimulus. This suggests that the diameter of these vessels may be insensitive to short-term increments or decrements in pressure. However, the occasional visual observation of halting flow in basal collecting venules, due to externally positive pressure onset or from middle ear contractions, suggests that some compression of the vessels probably occurs. It is possible that the vessel size change was too transitory to be observed, as pressure is relieved through the distension of the round window during the duration (0.25 s) of image averaging.

The magnitude of the vascular dilation with breathing of 5% CO_2 in oxygen was the largest equivalent diameter change seen for any tested stimulus. While the change in diameter seems small (only 0.5 μm for a vessel of average size), this translates to more than 16% reduction in vascular resistance. Much of the increase in cochlear blood flow (about 20%) for this stimulus could be attributed to the decrease in vascular

resistance because flow is proportional to the fourth power of diameter by Poiseuille's law. However, the absence of dilation when breathing 5% CO_2 in air, coupled with a greater increase in flow (50%) and small changes in heart rate and blood pressure suggests that the dominant factor controlling lateral wall blood flow remains elsewhere, probably in the modiolus.

A variety of results have been obtained relevant to vessel dilation under CO_2 stimulation. Perlman and Kimura (1955), using a relatively low optical-resolution system, found no in vivo diameter changes with rebreathing until after several minutes had passed. At that time, with the animal probably extremely acidotic, a dilation of 10% was seen. Dengerink et al. (1984) found no post-mortem diameter changes after breathing 10% CO_2 in oxygen. In these studies, even if no method-induced distortion of diameters had occurred, the maximum available resolution of the methods was probably insufficient to detect changes on the order seen in the current study. Dengerink et al. (1987), again using post-mortem techniques, did find diameter changes with administration of 10% CO_2 in air; however the exact way in which the stimulus was delivered (i.e., free or forced ventilation) was unclear, no comparisons were made with animals which were untreated or had recovered from the stimuli, and diameters were measured only in terms of RBC diameters with no calibrated measurements.

The dilation reported here, with CO_2 in oxygen but not found for CO_2 in air, is contrary to expectations of the reduction of CO_2-induced vasodilation by oxygen, as has been measured in other tissues (e.g., Duling, 1973). No satisfactory explanation is available at this time, so resolution of these partially conflicting results will require further study.

As noted earlier, once blood had ceased to flow in a vessel for more than about a minute, none of the tested stimuli seemed to be able to restore it. Therefore, this study offers little hope for breathing CO_2 mixtures of either type as a cure for existing lateral wall vascular obstructions, though 10% CO_2 in air was not tested. No conclusions can be drawn for modiolar vessels which may have considerably different properties. Alternatively, the activity seen in lateral wall vessels suggests that the use of CO_2 to prevent vascular shutdown does have merit. This is consistent with studies which have shown reduced temporary threshold shift (TTS) resulting from exposing guinea pigs to noise while breathing 5% CO_2 in oxygen (Brown et al., 1982), and hastened recovery from TTS in chinchillas with breathing of 5% CO_2 in oxygen following noise exposure (Joglekar et al., 1977). The persistence of dilation in the first turn relative to the third supports the notion that this area may be more easily overcome by metabolic stresses (e.g., Fechter et al., 1987).

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