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Real-time measurements of endogenous CO production from vascular cells using an ultrasensitive laser sensor

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Morimoto, Yuji, William Durante, David G. Lancaster, Jens Klattenhoff, and Frank K. Tittel. Real-time measurements of endogenous CO production from vascular cells using an ultrasensitive laser sensor. *Am J Physiol Heart Circ Physiol* 280: H483–H488, 2001.—Carbon monoxide (CO) has been implicated as a biological messenger molecule analogous to nitric oxide. A compact gas sensor based on a midinfrared laser absorption spectroscopy was developed for direct and real-time measurement of trace levels (in approximate pmol) of CO release by vascular cells. The midinfrared light is generated by difference frequency mixing of two nearinfrared lasers in a nonlinear optical crystal. A strong infrared absorption line of CO (4.61 μm) is chosen for convenient CO detection without interference from other gas species. The generation of CO from cultured vascular smooth muscle cells was detected every 20 s without any chemical modification to the CO. The sensitivity of the sensor reached 6.9 pmol CO. CO synthesis was measured from untreated control cells (0.25 nmol per 10^7 cells/h), sodium nitroprusside-treated cells (0.29 nmol per 10^7 cells/h), and hemin-treated cells (0.49 nmol per 10^7 cells/h). The sensor also detected decreases in CO production after the addition of the heme oxygenase (HO) inhibitor tin protoporphyrin-IX (from 0.49 to 0.02 nmol per 10^7 cells/h) and increases after the administration of the HO substrate hemin (from 0.27 to 0.64 nmol per 10^7 cells/h). These results demonstrate that midinfrared laser absorption spectroscopy is a useful technique for the noninvasive and real-time detection of trace levels of CO from biological tissues.

heme oxygenase; gas detection; difference frequency generation; vascular smooth muscle cells

CARBON MONOXIDE (CO) is a simple diatomic gas that may serve as an important cellular signaling molecule, much like nitric oxide (NO) (21). CO is generated as a byproduct of heme catabolism, in which heme oxygenase (HO) catalyzes the degradation of heme to biliverdin, iron, and CO (19). HO exists in at least three different isoforms that are products of distinct genes. Both the HO-2 and HO-3 isoforms are constitutively expressed and present in high levels in the brain (14, 15). In contrast, the HO-1 isotype is widely distributed and rapidly induced by its substrate, heme, and by various stress associated stimuli (14). In earlier studies (3, 6), our laboratory demonstrated that vascular smooth muscle cells (SMCs) express HO-1 and that both hemin (a stable form of heme) and NO stimulate HO-1 protein expression in these cells. A recent study (7) suggests that HO-1-catalyzed CO release by vascular cells may play a role in various cardiovascular disorders, including hypertension, ischemia-reperfusion, and endotoxin shock. However, a definitive role for CO in regulating cardiovascular function will require accurate measurements of CO synthesis during normal or pathological conditions.

Because the production of CO from biological tissues is extremely low (<10 nmol·mg protein^{-1}·h^{-1}), measurements of CO concentration have been limited to gas chromatography (4, 5, 10, 18, 22) and radioisotope counting (11, 13) techniques. Although these methods are highly sensitive, they cannot measure CO directly and require several time-consuming intermediate steps (>15 min) involving chemical reactions.

Infrared laser absorption spectroscopy is an attractive alternative approach for the detection of biological

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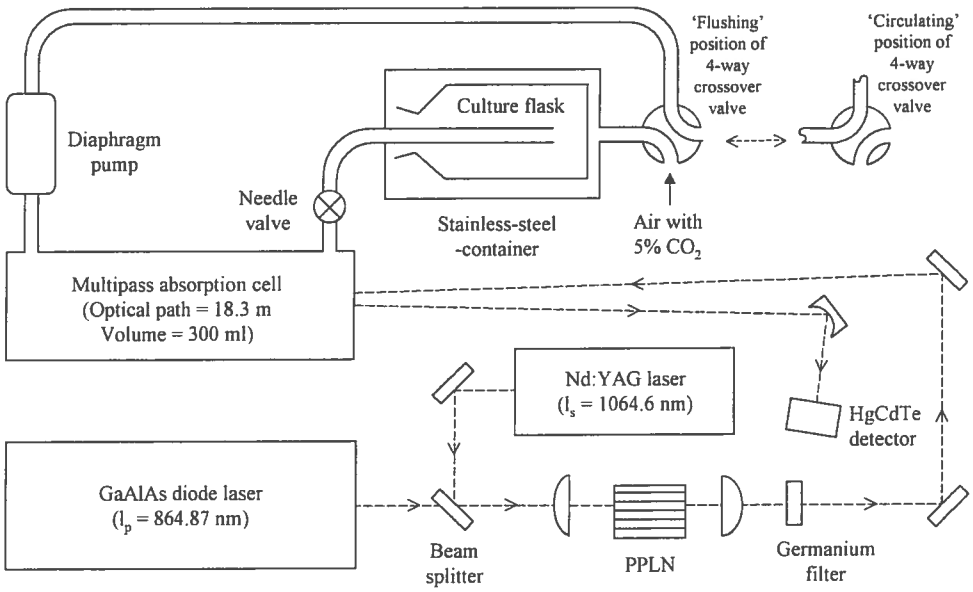

Fig. 1. Schematic of the difference frequency generation (DFG)-based gas sensor and gas flow system. PPLN, periodically poled lithium niobate crystal. GaAlAs, gallium aluminum arsenide laser; Nd: YAG, neodymium-yttrium aluminum garnet laser.

CO at the parts-per-billion (ppb) level in real time. Simple absorption measurements can detect CO directly and, unlike gas chromatography, avoids the addition of any chemicals that react with CO. Conventional infrared absorption spectroscopy that uses a Fourier-transformed infrared (FTIR) spectrometer is a well-known, reliable, and accurate system for trace gas detection. However, due to the low brightness of its light source and the mechanical displacement of mirrors required for wavelength scanning, its spectral resolution (typically ~0.5 cm^{-1}) and sensitivity [~1 parts per million (ppm) in the case of CO] are low, and its sampling time (~1 min) is longer compared with the laser absorption spectroscopic technique. The high sensitivity, high resolution, and fast response of laser absorption spectroscopy can be attributed to its inherent high spectral brightness and the availability of sensitive detection techniques. A tunable coherent light source developed for laser absorption spectroscopy (16, 20) generates midinfrared radiation at a wavelength of ~4.61 μm, where CO exhibits a characteristic fundamental rotational-vibrational absorption line. The midinfrared light is generated by difference frequency generation (DFG), where the outputs from two commercially available diode lasers that operate at room temperature and nearinfrared wavelengths are mixed in a periodically poled lithium niobate crystal. Recent advances in diode lasers and novel infrared nonlinear materials have permitted the development of this robust and compact DFG-based spectroscopic source. The sensor system is capable of detecting CO concentrations as low as 4.5 ppb, corresponding to 6.9 pmol CO/300 ml cell volume. The total measuring and processing time is ~20 s. The sensor does not suffer from interference by absorption lines of other gas species because of its high spectral resolution (~10^{-3} cm^{-1}) (16).

In the present study, we employed this DFG-based gas sensor to directly monitor the production of CO from intact living vascular SMCs.

MATERIAL AND METHODS

Cell cultures. Rat aortic SMCs were isolated by elastase and collagenase digestion of the rat thoracic aorta and were characterized according to morphological and immunological criteria, as previously described (8). Cells were propagated in minimum essential medium containing Earle's balanced salts, 5.6 mM glucose, 2 mM glutamine, 10% (vol/vol) heat-inactivated fetal calf serum, and 100 U/ml of penicillin and streptomycin. For experiments, subcultured cells were seeded onto 250-cm^3 volume flasks and used between passages 6 and 24. When the cells reached confluence (≈1 × 10^7 cells/flask), the culture media were replaced with serum-free media containing bovine serum albumin [0.1% (wt/vol)] for 24 h and then exposed to the various treatment regimens. Twenty-six flasks containing confluent cells were used in this study. The protein concentration of the cells was determined using the bicinchoninic acid method with serum albumin as the standard (17).

HO-1 protein analysis. Vascular SMCs were lysed in electrophoresis buffer [125 mmol Tris·HCl (pH 6.8), 12.5% glycerol, 2% SDS, and trace bromophenol blue] and boiled for 10 min. Proteins (20 μg) were then separated on 10% polyacrylamide gels by SDS-PAGE and transferred to nitrocellulose membranes at 100 V for 1 h. Membranes were blocked for 1 h in PBS containing 0.1% Tween 20 and 3% nonfat milk and then incubated with the HO-1 antibody (1:500 dilution; StressGen Biotechnologies, Collegeville, PA) in Tween 20 (0.1%) containing PBS for 1 h. The membrane was then washed in PBS and incubated for 1 h with anti-rabbit (1:7,500 dilution) horseradish peroxidase-conjugated antibody (Amersham, Arlington Heights, IL). After further washing the blots with PBS, we incubated the blots in commercial chemiluminescence reagents (Amersham) and exposed them to photographic film for 30 s according to the manufacturer's instructions.

Sensor configuration. Our present midinfrared sensor was tuned to the 2,169.198-cm^{-1} (4.61 μm), CO R(6)-branch absorption line because of its high line strength (4.44 × 10^{-19} cm/molecule) and relative freedom from interference by water and other gases. Details of the sensor were previously reported for the detection of CO (20), CH_4, and H_2CO (12). A schematic of the sensor is shown in Fig. 1. Tunable midinfrared radiation is generated by DFG, in which two input

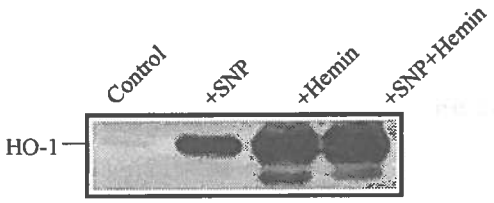

Fig. 2. Heme oxygenase (HO)-1 protein levels in vascular smooth mucle cells (SMCs). SMCs were treated with sodium nitroprusside (SNP; 1 mM), hemin (20 μM), or the combination of SNP (1 mM) + hemin (20 μM) for 24 h and then analyzed for HO-1 protein by Western blotting. Similar findings were observed in 3 separate experiments.

pump beams at wavelengths designated as a pump wave (λ_p; 864.87 nm) and signal wave (λ_s; 1,064.6 nm) were combined in a periodically poled lithium niobate crystal, resulting in the generation of an output wave at a wavelength $\lambda_i = (1/\lambda_p - 1/\lambda_s)^{-1}$; 4,610.0 nm. The DFG midinfrared beam was then directed through an 18-m path length multipass absorption cell (300 ml volume) (model 5611, New Focus, Santa Clara, CA), which was used to increase the measured optical absorption by CO. The DFG beam was then focused onto a thermoelectrically cooled HgCdTe detector. The detected signal with a spectral range of 0.241 cm^{-1} was averaged over 500 sweeps. The result was updated every 20 s for a spectroscopic measurement. For an accurate calibration of the CO levels, a 149.3 ppb calibrated CO mixture in air (prepared by Scott-Marrin, Riverside, CA) was used.

CO measurement. CO produced from vascular SMCs was measured using the above-mentioned sensor and a gas flow system (Fig. 1). An uncapped 250-cm^3 flask containing SMCs was transferred into the stainless steel container with a volume of 725 cm^3. The inside of this container was maintained at 37°C using a temperature-controlled heating pad. When a four-way crossover valve was set at the "flushing" position, the gases of both the container and the multipass absorption cell were purged with an air plus 5% CO_2 gas mixture with a CO concentration of 450 ppb prepared by Scott Specialty Gases (Plumsteadville, PA) for 20 min. The forced ventilation was impelled by a diaphragm pump (Type N84.3, KNF Neuberger, Trenton, NJ) at a flow rate of 120–130 ml/min to allow the CO concentration in the gas flow system to be 450 ppb. The 5% CO_2 was necessary to maintain the pH (7.4) of the culture medium and cell viability. The combination of the diaphragm pump and a needle valve enabled us to maintain the inside pressure of the multipass absorption cell at 100 Torr to increase the spectral sensitivity and that of the stainless steel container at 760 Torr. After ventilation, we switched the four-way crossover valve to a "circulating" position, which permitted the inside gases of the stainless steel container and the multipass absorption cell to be continuously recirculated in the closed gas flow system. CO inside the multipass absorption cell was measured by the midinfrared laser sensor every 20 s. The CO concentration at time = 0 was always 450 ppb with the standard deviation of 4.5 ppb, corresponding to 6.9 pmol CO/300 ml cell volume. The standard deviation of 4.5 ppb in CO concentration was calculated from successive 360 data, each of which was measured from the ventilation gas containing 450 ppb CO.

The sensor is capable of detecting CO ranging from 100 to 9,000 ppb with 0.6% accuracy (20). Interference from N_2, O_2, or CO_2 is not observed.

Statistical analysis. Data are presented as means ± SE. Statistical analysis was performed using a Kruskal-Wallis nonparametric analysis of variance followed by a Scheffé's test multiple comparisons if a significant probability was reached. A level of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Consistent with our previous studies (3, 6), control untreated vascular SMCs express low levels of HO-1 protein (Fig. 2). However, incubation of SMCs with the NO donor sodium nitroprusside (SNP; 1 mM) for 24 h markedly elevated HO-1 protein levels. Similarly, treatment of SMCs with the HO-1 substrate hemin (20 μM) for 24 h induced the expression of the HO-1 protein. However, the induction by hemin was greater than that observed with SNP. The combined addition of SNP (1 mM) and hemin (20 μM) to SMCs resulted in the highest level of HO-1 expression.

In subsequent experiments, the release of CO from vascular SMCs was measured using the infrared laser gas sensor. Control untreated SMCs released low levels of CO (0.25 ±0.07 nmol per 10^7cells/h, $n = 8$). Interestingly, incubation of SMCs with SNP (1 mM) for 24 h failed to increase CO production, even though HO-1 protein was markedly elevated (Fig. 3). In contrast, hemin treatment (20 μM) for 24 h resulted in an approximately equal to twofold greater increase in CO production compared with untreated control SMCs (Fig. 3). The ability of hemin to stimulate CO synthesis may, in part, reflect the higher levels of HO-1 protein found in hemin-treated cells relative to SNP-treated SMCs. However, given the low levels of free heme (10^{-7}–10^{-8} M) and its compartmentalization within cells, exogenously administered hemin may also promote CO synthesis by providing SMCs with additional substrate for HO-1 metabolism (9). Treatment of SMCs with the combination of hemin (20 μM) and SNP (1 mM) for 24 h resulted in the highest levels of CO

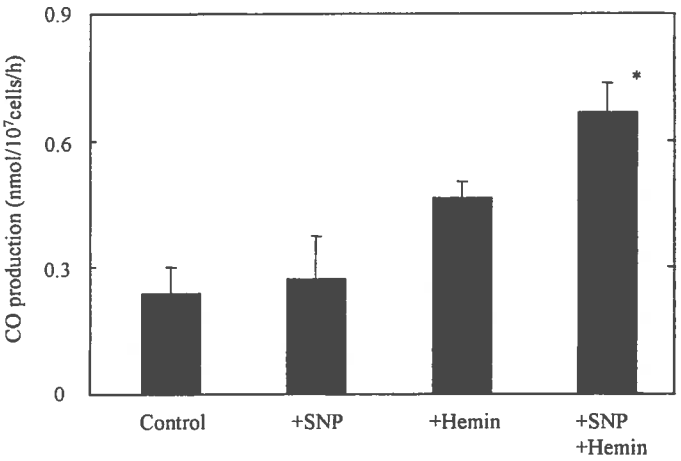

Fig. 3. Carbon monoxide (CO) release from vascular SMCs. SMCs were treated with SNP (1 mM), hemin (20 μM), or SNP (1 mM) + hemin (20 μM) for 24 h. After the exposure of SMCs to the various treatment regimens, we removed the media, and cells were washed with PBS (pH 7.4, 37°C) 3 times, and serum-free media were added. Each flask containing the cells was then transferred into a stainless steel container for gas detection. The net CO production was determined by subtracting the CO production in the flask containing serum-free media only. Results are the means ± SE of 3–8 experiments. *$P < 0.02$ compared with control.

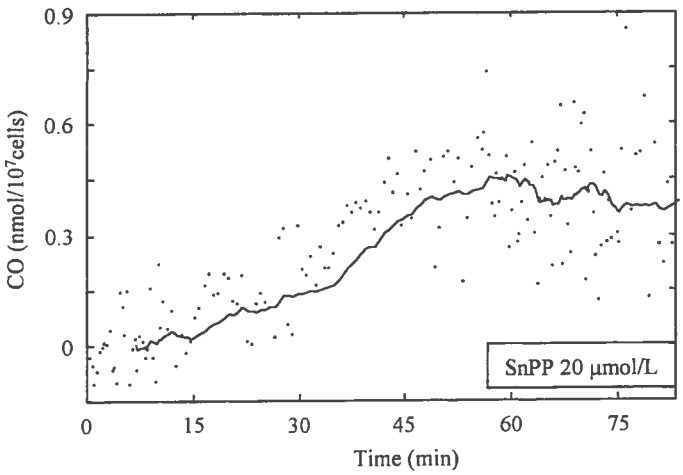

Fig. 4. Measurement of CO concentration inside the flask containing vascular SMCs treated with hemin (20 μM) for 24 h. CO release is inhibited immediately after the addition of the HO inhibitor, tin protoporphyrin-IX (SnPP; 20 μM), indicating that HO activity is responsible for the generation of CO. Each concentration point is updated every 20 s. The solid line indicates moving average over 20 points. Similar findings were observed in 3 separate experiments.

production (Fig. 3). This reflects the presence of high levels of HO-1 protein and substrate.

The DFG-based gas sensor enabled us to detect real-time changes in CO production. A time trace of the measured CO concentration is shown in Figs. 4 and 5. The gas inside the flask containing vascular SMCs was recirculated continuously through the optical absorption cell. During CO measurements, tin protoporphyrin-IX (SnPP) (20 μM), which is a potent HO inhibitor (23), was administered into the culture media using a microinjector (Fig. 4). The addition of SnPP blocked CO production from SMCs treated with 20 μM hemin for 24 h. The CO production rate significantly decreased from 0.49 ± 0.04 to 0.02 ± 0.10 nmol per 10^7cells/h ($P < 0.05$; $n = 3$). In the case of SMCs treated with both hemin (20 μM) and SNP (1 mM) for 24 h, the addition of SnPP (20 μM) also blocked CO production. The CO production rate after the addition of SnPP (0.24 ± 0.16 nmol per 10^7cells/h) was significantly less than that before the addition of SnPP (0.71 ± 0.07 nmol per 10^7cells/h, $P < 0.05$; $n = 3$). In contrast, the injection of the HO-1 substrate hemin (20 μM) to the flask enhanced CO synthesis (Fig. 5). CO synthesis from SMCs treated with 1 mM SNP for 24 h significantly increased from 0.27 ± 0.09 to 0.64 ± 0.12 nmol per 10^7cells/h ($P < 0.05$; $n = 3$) after the addition of hemin. These latter findings clearly demonstrate that HO is responsible for generating CO and that the formation of CO by vascular SMCs is substrate limited.

There are several studies that have reported the detection of CO release from biological tissues. However, all these studies experienced experimental difficulties in measuring CO due to limited CO production. To compare our results with these other studies, we converted our measurements from nanomoles per 10^7 cells per hour to nanomoles per milligrams of protein per hour (10^7 cells = 3.5 mg protein). The basal rate of CO production from SMCs was calculated to be 0.072 nmol·mg protein^{-1}·h^{-1}, which is comparable to a previous study that reported CO production rates of ~0.052 nmol·mg protein^{-1}·h^{-1} in cultured cells from the rat olfactory bulb (11). In contrast, CO production in broken cell fractions seems to be much higher than that found in intact cells. In the HO-enriched supernatant fraction of blood vessels, rates of CO formation of 1.4 nmol·mg protein^{-1}·h^{-1} were reported in rat aortic tissue (5), whereas CO rates of 0.50 nmol·mg protein^{-1}·h^{-1} were measured in the human mesenteric artery (10). Similarily, high levels of CO synthesis (2–6 nmol·mg protein^{-1}·h^{-1}) were detected in the supernatant fraction of guinea pig brain (4). The higher values of CO production found in studies employing cell fractions likely represent optimal idealized rates of CO synthesis because CO measurements were determined in the presence of saturating levels of substrate and cofactors.

CO can be detected by several methods, such as infrared absorption, colorimetry, electrochemical methods based on selective membranes, gas chromatography, and radioisotope counting. However, these methods, with the exception of gas chromatography and radioisotope counting, are not capable of detecting trace levels of CO below 1 nmol (roughly <1 ppm). There are currently three kinds of techniques based on gas chromatography for detecting sub-ppm levels of CO. One technique uses a flame ionization detector (10). CO is converted to methane by a methanator and then measured by the detector. The detection limit is 61 pmol CO/2 ml volume (2), corresponding to 750 ppb. Another technique is to use a helium ionization detector, which potentially has a CO sensitivity of 3 ppb. However, the carrier gas must be purified of water, oxygen, nitrogen, and carbon dioxide to a sub-ppb level. Hence, the detection limit for CO increases to 70 ppb (5.7 pmol CO/2 ml volume) in the presence of ambient air and humidity (1). The third technique employs

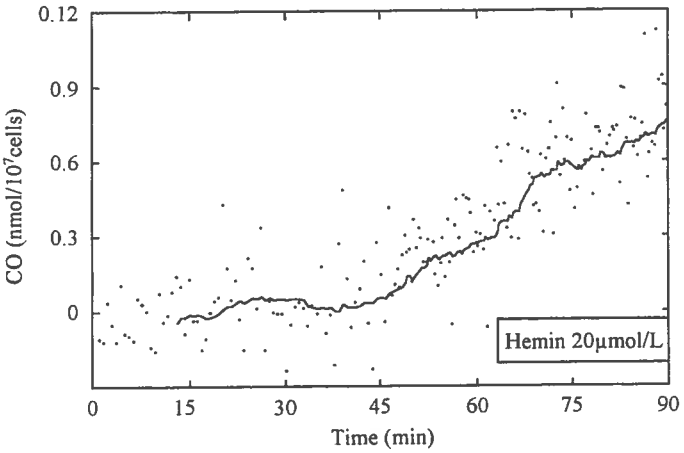

Fig. 5. Measurement of CO concentration inside the flask containing vascular SMCs treated with SNP (1 mM) for 24 h. CO release is enhanced immediately after the addition of the HO-1 substrate hemin (20 μM). Each concentration point is updated every 20 s. The solid line indicates moving average over 20 points. Similar findings were observed in 3 separate experiments.

atomic absorption by Hg vapor, which results from the chemical decomposition of HgO by CO (4, 5) and results in a high sensitivity; 1 pmol CO/2 ml volume (22), corresponding to 12 ppb. The radioisotope (^{14}C) method (11) is characterized by its high sensitivity with a picomole detection limit for CO. However, the intrinsic measurement process is time consuming because it involves the preincubation of biological samples with ^{14}C for up to 5 h. Moreover, the procedure is complicated because the generated gas must be drawn through five different traps to eliminate any contamination arising from $^{14}CO_2$ formation (11). The present method is based on a simple infrared absorption measurement, which has a high sensitivity (~4.5 ppb) and a relatively fast analysis time response (20 s). Moreover, this method does not suffer from interference by ambient air constituents, because the tunable DFG light source can access a specific strong rotational-vibrational CO absorption line at 2,169.198 cm^{-1}, which is free of interference.

The sensor enabled us to determine changes in CO concentration every 20 s with a single point sensitivity of 4.5 ppb CO, corresponding to 6.9 pmol CO/300 ml cell volume. However, the successive data fluctuated. The standard deviation of point-to-point values in Fig. 4 or 5 was calculated to ~140 pmol CO. It may be caused by dead volume, which does not contribute to the infrared absorption. The dead volume includes space inside the gas flow system, excluding the multipass absorption cell. Accordingly, regression analysis of successive points over 30 min was needed to obtain reliable CO production rates from 10^7 SMCs. If the multipass absorption cell is downsized to a 30-ml volume and the dead volume is reduced by 90%, the standard deviation of point-to-point values can be improved down to ~1.4 pmol CO.

This technique can be adapted to detect other biologically relevant gases, such as NO, which has a infrared absorption line at ~1,903 cm^{-1} (5.255 μm). Present sensitivity for NO is at the ~0.5-ppm level (unpublished data). This is because DFG conversion efficiency is low (<5% of the conversion at 4.61 μm) at 5.255 μm and the strength of the NO absorption is intrinsically much less than that of CO absorption at 2,169.198 cm^{-1}.

In summary, we report that real-time CO concentration measurements of biological tissue can be conveniently obtained by a midinfrared gas sensor. The sensor was originally developed to detect environmentally important atmospheric trace gases. However, on the basis of the data reported here, we demonstrate that infrared laser absorption spectroscopy using the DFG technique is a novel sensitive and selective method for direct and real-time measurement of CO production from vascular tissue.

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