Carbon Dioxide-Induced Anesthesia Results in a Rapid Increase in Plasma Levels of Vasopressin

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Brief anesthesia, such as after exposure to high levels of carbon dioxide, prior to decapitation is considered a more humane alternative for the euthanasia of rodents, compared with use of decapitation alone. Studies of the levels of certain stress hormones in plasma such as corticosterone and ACTH have supported the use of this method of euthanasia in endocrinological and molecular studies. In the current study, rats were briefly exposed to a chamber filled with carbon dioxide until recumbent (20–25 sec), immediately killed via decapitation, and trunk blood collected; findings were compared with rats killed via decapitation with no exposure to carbon dioxide. RIAs were used to measure arginine vasopressin (AVP) and ACTH immunoreactivity (ir) in plasma. Whereas ACTH-ir levels remained steady after brief exposure to carbon dioxide (in accordance with results of other investigators), AVP-ir levels were increased by more than an order of magnitude. These results were confirmed by quantitative capillary-liquid chromatography-mass spectrometry, indicating this observation of rapid increase in plasma AVP-ir levels is not due to nonspecific recognition by the antibody used in the RIA. Likewise, using capillary-liquid chromatography-mass spectrometry, we observed a rapid increase in plasma oxytocin levels after carbon dioxide exposure. These surprising findings have important implications for the design and interpretation of studies involving brief carbon dioxide exposure prior to decapitation as well as those with euthanasia resulting from carbon dioxide-induced asphyxiation. **(*Endocrinology* 150: 2934–2939, 2009)**

In the euthanization of animals, various factors must be considered, not the least of which is the humane treatment of the animals, but also the safety and comfort of the laboratory workers and the possible effects of the euthanasia method on any posthumous measurements that may be germane to the study (1). A common method of killing rodents is asphyxiation using carbon dioxide, which has been recommended for some time by the Universities Federation for Animal Welfare and the American Veterinary Medical Association (AVMA, Schaumburg, IL, http://www.avma.org/issues/animal_welfare/euthanasia.pdf; and see Ref. 2). In addition to use in asphyxiation-induced euthanasia, exposure to CO_2 for shorter time periods results in anesthesia. Exposure to CO_2 until the animal has become recumbent, followed by rapid decapitation is also a common method for euthanasia and is more suitable for endocrinological measurements (4). This method has been shown to have minimal effect on the levels of certain hormones related to stress response, with only a slight decrease in the plasma levels of corticosterone and prolactin, in rats (4). Similarly, exposure to CO_2 for 30 sec did not lead to changes in plasma levels of ACTH, although longer exposure did lead to elevations (5). The use of anesthetics, including CO_2, before decapitation, has been suggested as a more ethical method for euthanasia than decapitation alone (1, 4).

Since the initial enumeration of antidiuretic hormone [also known as arginine vasopressin (AVP)] and its ability to induce antidiuresis and alter arterial pressure, AVP has been intensely studied and characterized, and shown to have a role in various systems (6). A number of early pioneering studies focused on the release of AVP into plasma induced by pharmacological agents, including anesthetics (7). There have been studies focusing on the effects of CO_2 as an agent in euthanasia, either as an anesthetic before decapitation or as the primary inducer of death via asphyxiation, on several behavioral and endocrine parameters (4, 5, 8–11), but to our knowledge the effect on AVP at the time of

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.
Copyright © 2009 by The Endocrine Society
doi: 10.1210/en.2008-1408 Received October 6, 2008. Accepted January 30, 2009.
First Published Online February 12, 2009

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Abbreviations: AVP, Arginine vasopressin; HPA, hypothalamic-pituitary-adrenal; ms, mass spectrum.

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death after such brief exposure has not been previously addressed. More prolonged exposure to CO_2 as well as hypoxic conditions (low O_2) in hypercapnia/hypoxia studies has been addressed, and large increases in plasma vasopressin were observed (12–14). We report here a dramatic and very rapid increase in the plasma levels of AVP, as measured by RIA as well as capillary-liquid chromatography mass spectrometry, after exposure to CO_2, which serves as a cautionary note for the design and interpretation of studies incorporating CO_2 exposure for the purposes of euthanasia.

Materials and Methods

Animals

Male Fischer and Lewis rats (13–18 wk, 200–250 g) were used in all studies herein and purchased from Charles River Laboratories (Wilmington, MA). Rats were singly housed in stress-minimized rooms, with a 12-h light, 12-h dark cycle, and food and water were provided *ad libitum*. All animals were housed for at least 1 wk, with daily handling, before studies. Animals were housed and euthanized in a manner approved by The Rockefeller University Institutional Animal Care and Use Committee. Two groups of animals were studied. One was rapidly decapitated immediately upon removal from the home cage and trunk blood collected in ice-cold EDTA-coated tubes. The other group was placed in a chamber that was precharged with CO_2. The characteristics of the chamber were 18 in. (length) × 12 in. (width) × 18 in. (height) plexiglas, with a metal grid approximately 8 in. from the floor. Approximately 1 lb of dry ice pellets were placed on the floor of the chamber and allowed to sublime for at least 5 min with the chamber lid (equipped with small air holes for emission of ambient air) closed. The level of CO_2 in the chamber after 5 min preequilibration with dry ice was observed to be 100% (±10%), using Gastec calibrated ultrahigh-range carbon dioxide calorimetric detection tubes (Nextteq, Tampa, FL); a similar chamber setup for CO_2-induced recumbency has previously been reported to have a chamber level of 97% CO_2 (8). Animals were monitored until recumbent (defined as animal being prostrate and unresponsive), which for all rats occurred within a time range of 20–25 sec. The animals were then rapidly decapitated and trunk blood collected. Plasma was obtained by centrifuging the tubes at 3100 × *g* for 15 min at 4 C.

RIA

After collection, plasma was divided into fractions for mass spectrometry (see below), AVP RIA, and ACTH RIA. For ACTH, RIA was performed using untreated plasma, with a kit from Diasorin (Stillwater, MN), in a manner similar to our previous studies (15, 16). AVP RIA was performed using a kit from Phoenix Pharmaceuticals (Belmont, CA). Plasma (500 μl) was dried down using a vacuum centrifuge and reconstituted in binding buffer, and the RIA was then performed according to the manufacturer's instructions.

Capillary-liquid chromatography-mass spectrometry

Plasma (100 μl) was diluted 1:1 with 2% trifluoroacetic acid and filtered using centricon filters (molecular weight cutoff 10 kDa; Millipore, Billerica, MA) to remove proteins. Approximately 150 μl were recovered, with 50 μl remaining as the retentate. Capillary liquid chromatography was performed using in-house packed reversed-phase capillary columns. The analytical column (75 μM inner diameter, 5 cm length) was packed using PicoTip Emitter fused silica capillaries (New Objective, Woburn, MA) and 5 μM C18 beads, and the concentrating precolumn (75 μM inner diameter, 5 cm length) was packed using Integrafrit fused silica capillaries (New Objective) and 15 μM C18 beads. Then 150 μl of diluted, filtered plasma was loaded onto the precolumn, manually using a pressure bomb. A Pharmacia (Uppsala, Sweden) SMART system delivered a gradient of mobile phase A (1% acetic acid in water) and B (1% acetic acid in 80% acetonitrile/20% water): 0 min, 0% B, ramping to 100% B at 17 min, holding at 100% B until 23 min, returning to 0% B at 28 min, followed by a 15-min equilibration phase. The flow rate of the system was set to 30 μl/min, with a flow split resulting in column flow of approximately 100 nl/min, measured using graduated capillaries.

The capillary liquid chromatography system was interfaced with a LTQ ion trap mass spectrometer (Thermo Scientific, Waltham, MA). The mass spectrometric method was set up to record, serially, a mass spectrum (ms) scan, with a *m/z* range of 300-2000, followed by data-independent ms^2 and ms^3 scans for AVP (ms^2 precursor ion 1 *m/z* 543.0; ms^3 precursor ion 1 *m/z* 543.0, precursor ion 2 *m/z* 328.3), oxytocin (ms^2 precursor ion 1 *m/z* 504.5; ms^3 precursor ion 1 *m/z* 504.5, precursor ion 2 *m/z* 285.2), and ACTH (ms^2 precursor ion 1 *m/z* 764.7; ms^3 precursor ion 1 *m/z* 764.7, precursor ion 2 *m/z* 884.4). In all cases, the collision-induced activation level was set to 35.0% according to the manufacturer's nomenclature, with an activation time of 30.0 msec, and the window for precursor ion 1 was set to a width of $m/z = 2.0$, and the window for precursor ion 2 was set to a width of $m/z = 1.0$. The mass spectral data were analyzed using the Thermo Scientific software package Xcaliber. For standard runs, AVP was obtained from Phoenix Pharmaceuticals; oxytocin was synthesized by the Rockefeller University Proteomics Resource Center.

Results

RIA

RIAs were conducted for the measurement of ACTH and AVP in rat plasma, from both Fischer and Lewis rats (Fig. 1). Our laboratory has extensive experience with ACTH RIA (15, 16). The AVP RIA has not been used prior by our laboratory, although it has been used by many other groups. Whereas ACTH levels did not change on this brief exposure of the rats to CO_2, the levels of AVP increased drastically by greater than an order of magnitude ($P < 0.005$ for both strains of rats).

Mass spectrometry

The fragmentation pattern of AVP analysis was determined using AVP standards prepared in PBS immediately before loading onto the column for mass spectrometric analysis (Fig. 2A). For purposes of quantification of AVP, we used the integrated peak area of a mass chromatogram using ms^2 tandem mass spectra (precursor ion − vasopressin $[M+2H]^{+2} = 543.0$ *m/z*, ob-

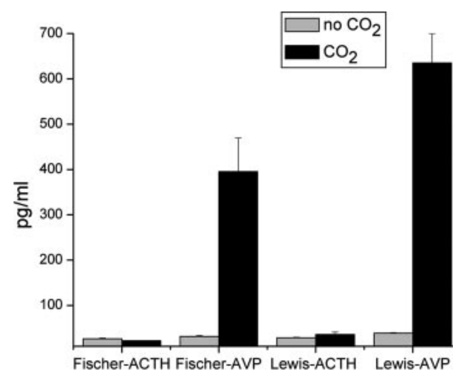

FIG. 1. Plasma from Lewis and Fischer rats, either decapitated without CO_2 (n = 3 for all groups) or exposed for 20–25 sec before decapitation, was used for RIAs of ACTH and AVP. Whereas AVP showed no change, plasma levels of AVP increased by more than 10-fold in each strain of rat.

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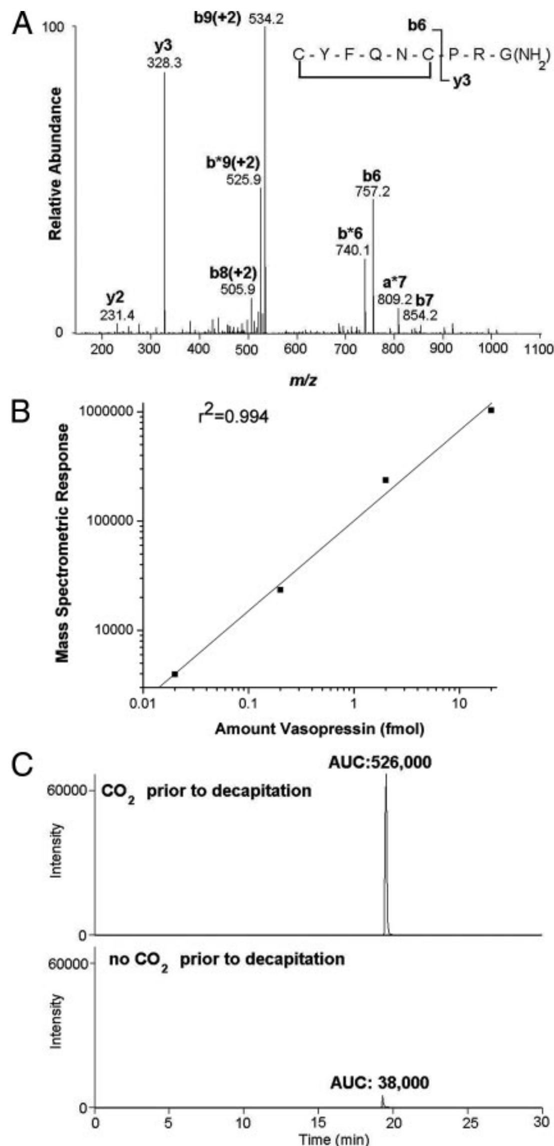

FIG. 2. Mass spectrometric detection of AVP. A, Fragmentation pattern of AVP: the most prominent fragments resulting from internal cleavage sites correspond to cleavage of the N-terminal cyclic moiety from the C-terminal tripeptide PRGamide, resulting in the y3 and b6 ions. B, AVP standards (0.02, 0.2, 2, and 20 fmol) were loaded in succession onto the capillary-liquid chromatography column and the eluent monitored via tandem mass spectrometry. Ms^2, mass chromatogram: doubly charged ion of AVP served as the precursor ion: m/z, 543.0 [ms^2 precursor ion 543.0 *m/z*; product ion monitoring of 328.2 *m/z*]. The base peak was set to correspond to the y3 product ion (328.3), and the resulting peaks were integrated using Xcaliber software (Thermo Scientific). The integrated peak areas are plotted *vs.* the amount of AVP loaded onto the column; a linear relationship for the amounts tested was found ($r^2 = 0.994$). C, Capillary-liquid chromatography-mass spectrometric detection of endogenous AVP in plasma. Ms^2 mass chromatograms [ms^2 precursor ion 543.0 *m/z*; product ion monitoring of 328.2 *m/z*] of plasma from representative rats exposed to CO_2 (*upper plot*) or decapitated without CO_2 exposure (*lower plot*), with the base peak set to the y3 ion, 328.3. Integration of the peaks is indicated by the resulting area under the curve (AUC). The ms^2 spectra were compared with the spectra from standard AVP (A) to confirm that the peaks shown correspond to authentic endogenous AVP.

serving y3 product ion = 328.3 *m/z*). A standard curve was generated over the range of 20 amol to 20 fmol of AVP in PBS by monitoring the integrated peak intensity from the ms^2 mass chromatogram; we observed a linear relationship of the integrated peak area over 3 orders of magnitude (Fig. 2B). Rats exposed to CO_2 gave a consistently higher integrated peak intensity corresponding to AVP compared with rats killed without CO_2, with representative chromatograms shown in Fig. 2C. Identification of the indicated peak as endogenous AVP was further verified by the ms^3 spectrum (data not shown).

In rats that were subject to CO_2 anesthesia before decapitation, oxytocin was unambiguously identified in plasma using mass spectrometry, based on the ms^2 and ms^3 fragmentation patterns (Fig. 3A). The background of the mass chromatogram using ms^2 tandem mass spectra (precursor ion − oxytocin $[M+2H]^{+2}$ = 504.5 *m/z*, observing y3 product ion = 285.2 *m/z*) was too high for use in the quantification of oxytocin in plasma; hence, we used ms^3 tandem mass spectra (precursor ion 1 − oxytocin $[M+2H]^{+2}$ = 504.5 *m/z*, precursor ion 2 − y3 fragment ion = 285.2 *m/z*, observing b2 product ion = 211.2 *m/z*). A standard curve of oxytocin in PBS indicated the linearity of integrated peak area of the ms^3 product ion 211.2 above 0.5 fmol (Fig. 3B). We observed oxytocin in the plasma of rats exposed to CO_2, but not in rats that were not exposed, indicating that the levels of oxytocin present basally in plasma are below the detection limits of the method (Fig. 3C). In comparison with the standard curve of oxytocin (Fig. 3B), the integrated peak areas observed from the plasma of rats exposed to CO_2 correspond to at least 10-fold greater than the detection limits, indicating the extent of increase in plasma oxytocin induced by CO_2 exposure is likely greater than 10-fold, in concordance with the increase in AVP.

Although we monitored ACTH using ms^2 and ms^3, we did not detect ACTH in either group of animals, indicating that the plasma levels of ACTH are below the detection limits of the capillary-liquid chromatography-mass spectrometric method (data not shown).

Discussion

In our laboratory, investigating stress responsivity in drug abuse, we have been using CO_2 before decapitation for a number of years in our studies of rodent models of drug abuse, with measurements of plasma levels of hormones such as corticosterone and ACTH as common end points (15, 16). Recently we observed that after exposure to CO_2, the levels of vasopressin in plasma were considerably higher than otherwise unprovoked levels in plasma reported in the literature, which are typically in the range of 0.5 to 5 pM (17–19). We therefore compared the effects of CO_2 exposure *vs.* the absence of CO_2 exposure to investigate this discrepancy. The rapidity and extent of AVP plasma increase are very dramatic and were unanticipated.

The use of RIA to measure AVP in plasma has a long history and has been used to show perturbations of AVP levels in plasma due to a variety of stimuli: pharmacological, cardiovascular, osmotic, *etc.* (20). The surprising results presented here, however, raise the possibility of artifactual cross-reactivity. The use of mass spectrometry for the detection of endogenous AVP confirmed that there was indeed a large, dramatic increase in the plasma levels of AVP after brief CO_2 exposure (Fig. 2C). The

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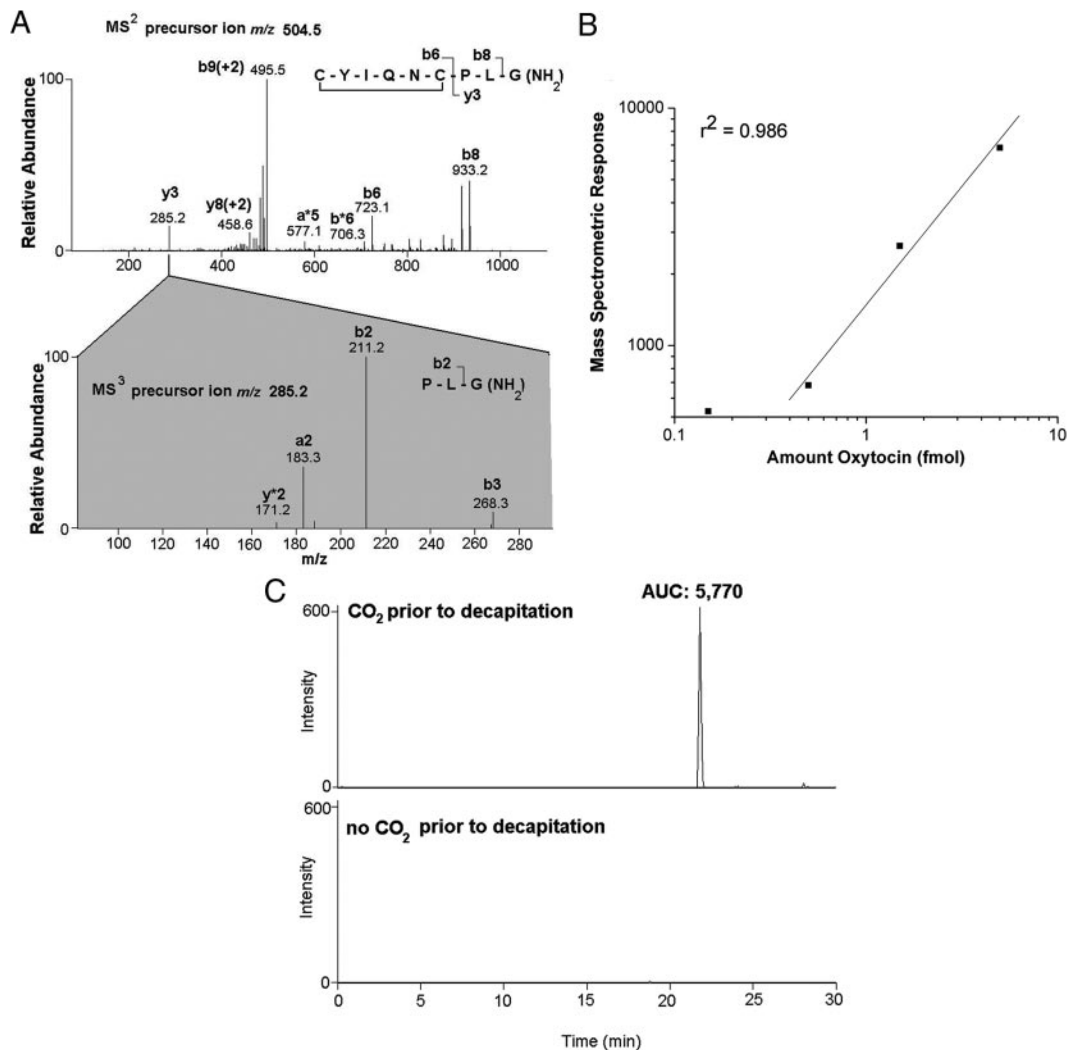

FIG. 3. Capillary-liquid chromatography-mass spectrometric detection of endogenous oxytocin in plasma. A, Ms^2 and ms^3 spectra of oxytocin in a representative rat exposed to CO_2, with *m/z* for precursor ions: 504.5 in the ms^2 spectrum (doubly charged oxytocin), 285.2 in the ms^3 spectrum (the y3 product ion of the oxytocin precursor ion), showing clear correspondence to endogenous oxytocin, with observed ions marked on the inset peptide backbone fragmentation diagram. B, Using oxytocin standard peptide in PBS, we determined the linearity and estimated the detection limits of oxytocin. With 5 fmol, the area under the curve (AUC) of the integrated peak of oxytocin ms^3 [precursor ion 1 = 504.5 *m/z*, doubly charged oxytocin; precursor ion 2 = 285.2 *m/z*, y3 product ion, product ion monitoring of 211.2 *m/z*], was 6820. The AUC was linear as the concentration was decreased an order of magnitude, to 0.5 (AUC of 0.5 fmol, 680) (r^2 = 0.986). Measurements of AUC less than 600 were not reliable, without correspondence to concentration and spectra typically lacking other fragment ions corresponding to the 285.2 product ion (A). Although the AUC for 0.15 fmol of oxytocin is plotted, it was not included in the regression line. C, Ms^3 chromatograms [precursor ion 1 = 504.5 *m/z*, doubly charged oxytocin; precursor ion 2 = 285.2 *m/z*, y3 product ion, product ion monitoring of 211.2 *m/z*], for representative rats exposed to CO_2 (*upper chromatogram*) or not exposed to CO_2 (*lower chromatogram*). There was no oxytocin detected, using ms^3 fragmentation criteria, in the absence of CO_2, whereas we observed oxytocin with AUC of 5770 after CO_2 exposure.

fragmentation of vasopressin and oxytocin (Figs. 2A and 3A) are similar to that previously observed by other investigators (21, 22). The standard curve (Fig. 2B) obtained for AVP present in PBS indicates that the integrated signal of the y3 ion (*m/z* = 328.3) in tandem mass spectra of AVP is a reliable measure of AVP levels. The curve demonstrates linearity of the integrated peak intensity of AVP in the ms^2 mass chromatogram greater than 3 orders of magnitude (also found to be linear in another recent mass spectrometric study of AVP) (21). Although relative levels of AVP can be determined, we note that the absolute levels of AVP in plasma cannot be discerned by comparison with the standard curve shown in Fig. 2B; the presence of other peptides in plasma are expected to alter the extent of vasopressin ionization and hence the peak intensities.

The use of mass spectrometry in this instance provided the additional advantage of allowing us to also determine the presence of oxytocin in the blood after CO_2 exposure. We did not have oxytocin antibodies or radioiodinated standards immediately available to perform RIA of oxytocin; the mass spectral parameters corresponding to oxytocin were included in the mass spectrometric method essentially as a hunch, given the relationship of oxytocin to vasopressin. Because the baseline levels of oxytocin were not detectable, the extent of increase cannot be accurately determined, although the results of a comparison of standards (Fig. 3B) indicate the oxytocin increase to be at least 10-fold. Although the basal plasma levels of oxytocin and AVP are generally comparable (19), the relative sensitivity of our method is sufficient for detection of basal AVP but not basal oxytocin.

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The demonstration of a large increase in plasma oxytocin levels strengthens the notion that CO_2 induces a significant change in the hormone levels of plasma and adds a further note of caution in the use of CO_2 in the euthanasia of animals. Note that in the case of oxytocin, ms^2 was not sufficient for detection of oxytocin above background, but ms^3 unambiguously shows the presence of this peptide after CO_2 exposure. The use of ms^3 for the detection of peptides has similarly been recently used for the detection of endogenous enkephalins (23). Further improvements in sample preparation, including the use of higher levels of plasma, will be investigated with an eye toward the detection of basal levels of oxytocin. No ACTH was observed in any sample using mass spectrometry, indicating the levels of ACTH were below the detection limits of mass spectrometry in both the absence and presence of CO_2 exposure, results not inconsistent with the results of the RIA for AVP.

Previous studies have also investigated hypothalamic-pituitary-adrenal (HPA) axis activation through the measurement of ACTH levels after CO_2 exposure. After 2 min of exposure to CO_2, ACTH levels are increased severalfold (5, 24). Hackbarth *et al.* (5), in a comparative study, with different times of CO_2 exposure, found that ACTH levels had not yet begun to rise after 30 sec of CO_2 exposure, in concordance with the results found here (Fig. 1). Several previous studies in our laboratory using similarly brief ($<$30 sec) CO_2-induced anesthesia before decapitation of rodents demonstrated plasma ACTH and corticosterone levels, which are likewise not consistent with pronounced HPA axis activation (15, 16, 25, 26), although it should be noted these studies did not directly compare animals with and without CO_2 exposure. It is possible that the delay in CO_2-induced ACTH increase is due to a requirement for prior release of an ACTH secretogogue, such as corticotrophin releasing factor; because AVP has been shown to be an ACTH secretogogue (27), it may be that the substantial increase in AVP levels observed here is the primary factor for the rise in ACTH levels observed after 2 min CO_2 exposure (5, 24).

Whereas the results of the current study were entirely unanticipated, there are reports of investigations that are in concordance with the AVP release observed herein. In addition to the hypoxia/hypercapnia studies in canines (13, 14), which were done using longer exposures, brief CO_2 exposure in rats, although generally found to have modest effects on hormonal levels (4), results in significant analgesia, for up to an hour after CO_2 exposure (10). The mediator of this analgesia was not determined, but it was shown to be both nonopioidergic, because it was not blocked by naloxone, and dependent on the presence of an intact pituitary gland, because it was blocked by neurohypophysectomy. Because intravenous AVP at high doses in rats induces nonopioidergic analgesia, also for up to an hour (28), the present results suggest that the release of high levels of endogenous vasopressin possibly serves as the mediator of the CO_2-induced analgesia.

The investigation of CO_2 effects on plasma hormone levels in man (29–34) correspond to some degree with the findings in animals, although the extent of exposure and the time course of plasma sampling are necessarily different. In a study by Kaye *et al.* (31), exposure to a single breath of 35% CO_2 resulted in a significant increase in ACTH in as little as 2 min, similarly to that described in rodents after longer and higher levels of CO_2 (5, 24), although the increase is much more modest, as should be expected. These investigators did not find an increase in AVP at the 2-min time point. Certainly there are a number of differences between their study and the current one, including CO_2 concentration, exposure duration, sampling time, and species studied, which likely accounts for this difference.

Our finding of a dramatic CO_2-induced increase in AVP and oxytocin has important implications for the use of CO_2 in methods of euthanasia in studies involving endocrinological measurements. Currently we can but speculate on the potential mechanisms underlying this rapid CO_2-induced increase in AVP and CO_2. The regulatory mechanism may involve mediation by glutamate (35), catecholamines (36, 37), serotonin (30, 38), or acetylcholine (39), possibly after preliminary activation of carotid chemoreceptors (40). It is possible that hypotension plays a role. Future investigations into the mechanism of CO_2-induced increases in AVP may yield insights into other effects of CO_2, such as panic-associated behaviors and later increases in HPA activity (29–34, 36).

Acknowledgments

The authors thank Dr. Yan Zhou and Dr. Eduardo Butelman for helpful conversations regarding the findings of this study as well as Dr. Beatrix Ueberheid for valuable guidance regarding the capillary-liquid chromatography mass spectrometric instrumentation.

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This work was supported by National Institutes of Health-National Institute on Drug Abuse Grant P60-DA05130 (to M.J.K.) and National Institutes of Health-National Center for Research Resources Grant RR00862 (to B.T.C.).

Disclosure Summary: The authors have nothing to disclose.

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