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Radiolabel validation of cortisol in the hair of rhesus monkeys

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ABSTRACT

Analysis of cortisol in hair has become a widespread tool for assessment of hypothalamic-pituitary-adrenal (HPA) axis activity because of its ease of collection and its ability to provide cumulative data over a period of months. In order to meaningfully interpret hair cortisol, however a direct validation by radio-metabolism is required to understand cortisol incorporation into hair. Tritiated [^{3}H]-cortisol was IV administered to adult rhesus monkeys to determine 1) if [^{3}H] is found in the hair after injection of [^{3}H]-cortisol, 2) the growth rate of hair and 3) the form in which cortisol is found in hair. Samples of hair were collected from newly and previously shaved patches at 14-days and 28-days after [^{3}H]-cortisol injection. Hair was processed by external wash, grinding, and hormone extractions. Samples were separated by high-performance liquid chromatography (HPLC) and fractions were collected and radioactivity assessed. We found [^{3}H] incorporated into the hair by the 14-day hair collection and no new radioactivity was found by the 28-day collection. Individual hair growth rate was highly variable between monkeys, indicating that the between-subject hair growth patterns were not consistent. Importantly, for the first time, we showed that systemically administered [^{3}H]-cortisol was found in the hair as [^{3}H]-cortisol and [^{3}H]-cortisone, as well as other glucocorticoid metabolites.

1. Introduction

Hair is becoming an increasingly popular matrix to measure cortisol as a biomarker of stress. It has been used in a diverse range of topics including: autism (Ogawa et al., 2017), the impact of asthma in children (Kamps et al., 2014), socioeconomic status (Vliegenthart et al., 2016), sleep (Maurer et al., 2016) as well as the association of hair cortisol with disease (e.g. Pereg et al., 2013). It has also been used to assess steroid hormones *in utero* from neonates, a measure that is only possible in hair, and more recently nails (Tegethoff et al., 2011), without invasive sampling.

Measurement of cortisol in traditional matrices - blood, saliva, and urine - is validated and well-established, but presents challenges. For example, in order to obtain long-term information about glucocorticoid function, repeated sampling is required which can be difficult for pediatric and other vulnerable populations where access is limited. In addition, the invasiveness of the sampling can also present an ethical issue. Measurement of cortisol in hair has many benefits: it is non-invasive, provides long-term information and a retrospective assessment, it is easy to transport and store, and avoids problems of non-adherence (Novak et al., 2013; Stalder and Kirschbaum, 2012). Indeed, from 2007 to 2017, the number of publications that have used hair to measure cortisol has increased over 7-fold. However, there are a number of issues that need to be addressed for this method to be considered reliable for measuring long-term levels of cortisol.

There are many uncertainties concerning how cortisol enters the hair and what factors may affect hormone disposition and residence in hair. Five potential mechanisms of incorporation, derived from drug incorporation studies, have been proposed (Cone, 1996; Harkey, 1993): (1) passive diffusion into hair from the bloodstream (central mechanism), (2) incorporation via sweat or, (3) sebum, (4) external sources and (5) cortisol produced locally in the hair follicle. The majority of studies have assumed that mechanism (1), diffusion of cortisol into the hair from the bloodstream is the predominant pathway of incorporation and therefore hair cortisol is a direct measure of central hypothalamic-pituitary-adrenal (HPA) axis activity.

Indirect support for this mechanism has come from a number of important studies in humans and non-human primates. In humans, D'Anna Hernandez et al. correlated area under the curve of 6-days, 3-times daily saliva collection with hair cortisol during each trimester of pregnancy (D'Anna-Hernandez et al., 2011). They showed that in the 2nd and 3rd trimesters there was moderate correlation between saliva and hair cortisol demonstrating concordance between the two biological matrices. Short et al., demonstrated that the area under the curve from a 3 times daily salivary cortisol measure was significantly correlated with hair cortisol levels (Short et al., 2016). A key factor of both of

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these studies was that hair was cut at baseline and from the same place at testing, ensuring all hair evaluated was grown during the evaluative period. Similar to the results found in the human studies, in rhesus monkeys, Davenport et al. demonstrated that there was a moderate positive relationship between saliva and hair cortisol in response to a relocation stress (Davenport et al., 2006). While these indirect studies demonstrated that there are relationships between biological matrices that are known to represent circulating cortisol and hair, the gold standard for obtaining direct support for the central mechanism is with a labelled-metabolism study (Penner et al., 2012). A labeled form of the analyte of interest is administered to a subject and then can be traced through the body by measurement of the label from samples collected. This technique has frequently been used for determination of hormone metabolites in urine and feces (e.g., Ziegler et al., 1989). Only one such study has been conducted to determine if tritium labeled ([^{3}H]) cortisol injected into guinea pigs was detected in the hair (Keckeis et al., 2012). The authors found that the majority of the radiation was excreted, but a small proportion was found in the hair in the form of cortisone, a metabolite of cortisol. They also found high levels of unlabeled cortisol in the hair suggesting that cortisol measured in the hair could be from other sources, such as from local production in the follicle.

Therefore, the results of indirect and direct studies in support of the central mechanism are mixed; measures of cortisol from other matrices are correlated with hair cortisol as well as events that are considered stressful, but, the direct evidence suggests that hair cortisol is not a good measure of circulating cortisol in a guinea pig. This discrepancy must be resolved by a radio-metabolism study of hair cortisol in a species that is biologically similar to a human in order to determine if hair cortisol is a valid measure of circulating cortisol and central HPA axis activity.

Among other issues concerning hair cortisol methodology is the rate of hair growth. The growth rate of scalp hair in humans is often assumed to be 1 cm/month and many research studies have capitalized on this growth rate by segmenting the hair to capture hair cortisol levels during specific periods of time. Examination of the original literature for the human hair growth rate shows that the range is variable from 0.6 to 3.35 cm/month (Giovanoli-Jakubczak and Berg, 1974; Harkey, 1993) suggesting that it may not be appropriate to segment the hair to measure cortisol in discrete windows of time. The current radiolabel study will enable us to determine the individual hair growth rate. We can then use that information to cut the hair to determine if the radiolabel was captured in that segment.

While there are benefits of analyzing cortisol in hair and there is already a large body of research that utilize the technique, there are a number of issues that need resolution in order to ensure that hair cortisol is being used in a biologically relevant manner. In this study we will IV inject radiolabeled cortisol to rhesus monkeys to determine: 1) if [^{3}H] is found in the hair after injection of [^{3}H]-cortisol to determine the importance of a central pathway of incorporation, 2) the growth rate of hair and whether hair can be segmented to represent windows of hair cortisol exposure and 3) the form in which cortisol is found in hair to measure which analyte in hair is representative of circulating cortisol. Rhesus monkeys are an ideal model species for the endocrinology of humans and therefore the data obtained from this study can be readily translated (Bourget et al., 1988; Mottet et al., 1987; Pal, 1979).

2. Methods

2.1. Animals and housing

Animals were maintained in accordance to guidelines for the ethical care and treatment of animals as approved by the Institutional Animal Care and Use Committee of the Graduate School of the University of Wisconsin-Madison. Seven male adult (age range: 4–11, mean = 6.3 years; weight range: 7.2–14.9, mean = 10.8 kg) rhesus macaques from the colony at the Wisconsin National Primate Research Center (WNPRC) were used for evaluation of incorporation of [^{3}H]-cortisol into hair. Three days prior to [^{3}H]-cortisol administration the monkeys were acclimated to the experimental room that was isolated from the rest of the colony. The monkeys were singly-housed for containment of radioactivity. All males were housed simultaneously in the same room to provide visual and auditory stimulation during the experimental period. The monkeys remained in the experimental housing until the radioactivity was considered to be near basal levels and then returned to their home cages 4 days after [^{3}H]-cortisol administration. Monkeys were on a 12:12 light-dark cycle (ON: 0600, OFF: 1800), and were fed twice daily with commercial chow (Harlan Teklad #2050, 20% protein Primate Diet, Madison, WI) and given a variety of fruit in the afternoons. Water was available ad-libitum. Housing rooms were maintained at 65–75 °F, 30–70% humidity. In addition, foraging activities and physical environmental enrichment were provided when monkeys were in their home cages.

2.2. Administration of radiolabeled hormone and sampling of urine, feces and hair

Radiolabel experiments were approved by the Radiation Safety committee at the University of Wisconsin-Madison. Ethanol, absolute, and scintillation fluid (ScinitSafe Econo 2 Cocktail) was purchased from Sigma (St. Louis, MO). Tritium labeled cortisol (Cortisol [1,2,6,7-H(N)], 50–90 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Monkeys were anesthetized (Ketamine anesthesia,10 mg/kg i.m.) for IV delivery of the tritium labeled cortisol. Two 6 x 4.5 cm sections of hair from the upper back were carefully shaved to the skin with grooming clippers and used for baseline cortisol levels.

2.2.1. Experiment 1

The initial [^{3}H]-cortisol experiment was conducted in 3 monkeys. Following the baseline hair shave, 83.3 μCi [^{3}H]-cortisol in 5 mL of sterile saline was administered IV into the saphenous vein. After [^{3}H]-cortisol administration, the IV apparatus, syringe and the vial containing the radiolabel was rinsed twice with ethanol and the residual radioactivity was counted and subtracted from the pre-injection total to determine the precise amount of radioactivity administered.

Urine and fecal samples were collected prior to [^{3}H]-cortisol administration for evaluation of baseline levels of tritium and then collected every 24-hours for the following consecutive 4 days and counted for tritium until the radiation levels were considered close to normal and the monkeys could return to their home cages. Hair was collected 30 and 60 days post-injection from a previously shaved area (re-shave) and from a new area (new-shave) on the upper back by the procedure described above. These times were chosen to reflect hair cortisol as an assessment of cumulative long-term circulating cortisol levels.

2.2.2. Experiment 2

This experiment was conducted in 4 monkeys. Based on the results of Experiment 1, we determined that in order to detect [^{3}H]- in the hair, the injection amount needed to be increased to 250 μCi [^{3}H]-cortisol per monkey. We also determined that hair could be collected earlier than 30 days based on the hair growth results so collection times were changed to 14 and 28 days (Fig. 1). Urine and feces collection and measurement of radiation lost in the IV apparatus and vials were measured as in Experiment 1.

2.3. Extraction and determination of radioactivity in urine, feces and hair

Tritium was measured using a 6895 Beta Trac TM Analytic liquid scintillation spectrophotometer (Arlington Heights, IL). Quench correction was used to determine disintegrations per minute (DPM) in samples which was converted to μCi for analysis. Each sample was counted for a maximum of 20 min. Background counts (ethanol in

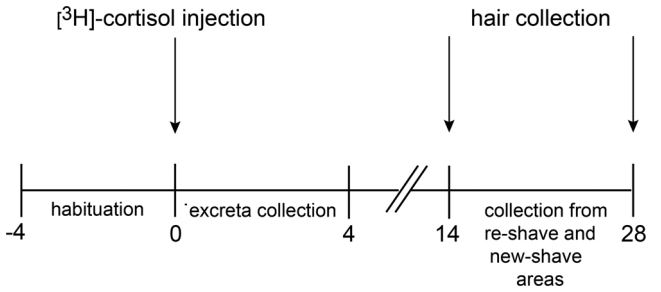

Fig. 1. Schematic diagram of experimental protocol for [^{3}H]-cortisol administration and excreta and hair collection.

scintillation fluid) was also measured and subtracted from counts measured in the samples. Background counts (ethanol in scintillation fluid) was also measured and subtracted from counts measured in the samples.

2.3.1. Urine

Within 1-hour after urine collections, 1 mL of urine in 20 mL scintillation cocktail was counted for tritium and corrected for volume to determine the amount of [^{3}H] excreted via urine.

2.3.2. Feces

Fecal samples were weighed (1 g) and mixed with 1 mL methanol. A portion of the methanol extract was added to 20 mL scintillation fluid and counted and then corrected for total fecal weight to determine the amount of [^{3}H] excreted via feces.

2.3.3. Hair

Hair was measured, cut (for new-shaved regions), weighed and washed twice with isopropanol to remove any external contamination as per Davenport et al. (2006). This method demonstrated that isopropanol did not remove cortisol from the interior of the hair shaft (Davenport et al., 2006). The 2 wash solutions were combined and measured for radioactivity to determine how much [^{3}H] was on the outside of the hair shaft. Radiolabeled cortisol within the hair matrix was extracted by grinding the hair using a ball grinder (Verder Scientific, Newtown PA), 2 ml methanol added and incubated with inversion rotation overnight. After incubation, the tubes were centrifuged, and 100 u L of the supernatant was used to assess total tritium counts in the sample.

2.4. Characterization of 3H-cortisol and metabolites in hair samples

The remainder of the methanol extracts from the ground hair were subjected to reversed-phase HPLC with UV detection (Beckman Coulter) using a Luna 5 µM Kinetex C18 column (Phenomenex, Torrance, CA). Isocratic flow of 30% acetonitrile and 70% water was used for 20 min and fractions were collected in 1-minute increments using a Fractionator. The fractions were transferred to vials with scintillation fluid to determine the fraction(s) in which [^{3}H] was detected. Cortisol and cortisol metabolite standards were run through the same chromatography and the elution profile of the standard was compared with the fractions containing [^{3}H]. Cortisol and cortisone standards were purchased from Cerilliant (Round Rock, TX). All other cortisol metabolites (α-cortolone, β-cortolone, α-cortol, tetrahydrocortisol, tetrahydrocortisone, 5β-dihydrocortisol, 5-dihydrocortisone, 20-dihydrocortisol, 20-dihydrocortisol, 11-deoxycortisol, 6-hydroxycortisol) were purchased from Steraloids (Newport, RI).-dihydrocortisol, 5β-dihydrocortisone, 20α-dihydrocortisol, 20β-dihydrocortisol, 11-deoxycortisol, 6β-hydroxycortisol) were purchased from Steraloids (Newport, RI).

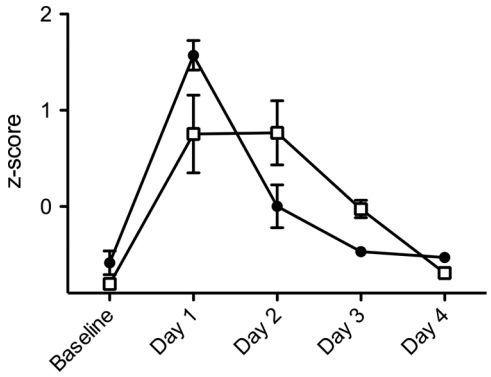

Fig. 2. Z-scores of [^{3}H] counts (µCi) in urine (closed circles) and feces (open squares) in rhesus monkeys after [^{3}H]-cortisol injection.

2.5. Data analysis

Samples from monkeys from experiment 1 and 2 were used for determining [^{3}H] excretion in urine and feces, [^{3}H] residue remaining in the vial and IV injection apparatus, and for hair growth data (n = 7). Excretion data was presented as z scores due to the different amount of [^{3}H] administered in each experiment (Fig. 2). Tritium measurement in the vials and injection apparatus was presented as µCi and hair growth data were presented in cm at 14 days (experiment 2, n = 4) and 30 days (experiments 1 and 2, n = 7). Determination of the amount of [^{3}H] and the form in hair was calculated in the monkeys whose hair grew for re-shave (n = 2). Total counts in the hair and the hair wash are presented as µCi. Radioactivity measurement in the HPLC fractions was analyzed as percentage of total radioactivity. Results in the text are presented as mean ± standard deviation. All data was analyzed in Prism (Version 5, GraphPad Software, La Jolla, CA) and SPSS (IBM SPSS for Macintosh, Version 23, Armonk, NY).

3. Results

3.1. [^{3}H] recovery

Distribution of [^{3}H] for each monkey is presented in Table 1. In experiment 1, after accounting for excretion and loss of [^{3}H] in the injection process, only 35.08 ± 7.2 µCi was left in the body for possible incorporation into hair which did not permit detection. In experiment 2, 118.53 ± 12.9 µCi was left in the body, which did permit detection in the hair. The majority of the radiolabel was excreted via urine on the day after injection as was expected for [^{3}H]-cortisol (Table 1 and Fig. 2). Urine and feces were monitored for 4 days after injection at which point the [^{3}H] had returned to baseline levels.

Table 1
Distribution of [^{3}H]-cortisol in each monkey in experiments 1 and 2. Data presented as µCi (%).

Amount excreted urine	Amount excreted feces	Amount lost in vial/ injection apparatus	Amount Left in Body
Experiment 1 (Starting amount: 83.3 µCi)			
53.84 (64.6)	0.27 (0.3)	2.02 (2.4)	27.17 (32.6)
38.99 (46.8)	0.28 (0.3)	2.76 (3.3)	41.27 (49.5)
43.88 (52.7)	0.22 (0.3)	2.40 (2.9)	36.80 (44.2)
Experiment 2 (Starting amount: 250 µCi)			
116.62 (46.6)	1.13 (0.5)	2.96 (1.2)	129.29 (51.7)
148.88 (59.6)	0.61 (0.2)	0.49 (0.2)	100.02 (40.0)
116.59 (46.6)	0.65 (0.3)	3.15 (1.3)	120.39 (48.2)
122.92 (49.1)	0.41 (0.2)	2.24 (0.9)	124.43 (49.8)

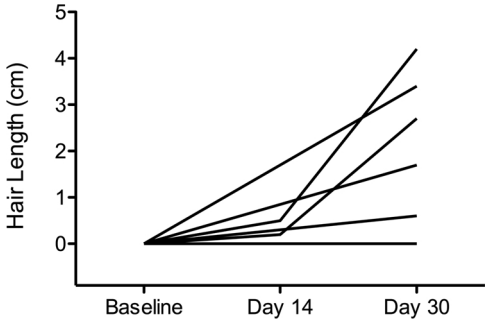

Fig. 3. Hair length in rhesus monkeys after baseline hair shave.

3.2. Time to [^{3}H] incorporation in hair

In experiment 1, hair was shaved at day 35 post [^{3}H]-cortisol injection from an area that had previously been shaved (re-shave) and a new area (new-shave). During experiment 1 we determined that there was sufficient hair growth to collect hair earlier than day 35 so for experiment 2, hair was re-shaved from one area on day 14 and another area on day 28. New-shaves were also collected at those time points.

The hair growth rate was variable between monkeys and ranged from no growth to 4.2 cm over 30 days. The mean was 1.8 cm in 30 days (Fig. 3).

The re-shaved hair was washed, ground and extracted and [^{3}H] was detected in both monkeys at days 14 and 28. The amount of [^{3}H] measured in each sample demonstrated that by day 14 post-injection all of the [^{3}H] had been incorporated into hair. By day 28 it appeared that no new [^{3}H] was being incorporated and the counts of [^{3}H]/mg were decreased due to increased hair growth (Table 2).

As the hair growth rate of the individual monkeys was known, new-shave hair was cut to represent the length that grew since the [^{3}H]-cortisol injection. There was no [^{3}H] detected in any of the cut samples (Table 2).

Prior to processing for measurement of [^{3}H] incorporated into the hair, hair was washed to remove any external contamination and [^{3}H] was detected at day 14 from the re-shave (2.29e-007 ± 8.1e-008 μCi) and new-shave (9.10e-008 ± 4.9e-008 μCi) areas.

3.3. Characterization of [^{3}H] in hair

The re-shave hair from day 14 in experiment 2 was separated by HPLC-UV to determine the form of the [^{3}H] incorporated into hair (Fig. 4). This was compared with the elution profile of the cortisol and cortisone standards. Tritium was detected in the hair fraction that corresponded to the positions of the cortisol and cortisone standards (Fig. 4), demonstrating that radiolabeled cortisol from circulation was incorporated into hair as cortisol (20.67 ± 2.5%) and cortisone (17.58 ± 8.1%). Radioactivity was also detected in fractions 1, 3, 7 and 11 which did not correspond to the positions of any other cortisol metabolites tested (see methods for complete list of metabolites).

Table 2
Hair growth and ^{3}H counts (μCi e-7/mg hair) in hair of monkeys centrally administered [^{3}H]-cortisol. ND (not detected).

Monkey	Day 14 Re-shaved area 3H counts	Day 28 Re-shaved area 3H counts	Day 14 New-shaved area 3H counts
1	6.3	1.1	ND
2	7.2	0.9	ND
Mean (SD)	6.75 (0.6)	1.0 (0.1)	–

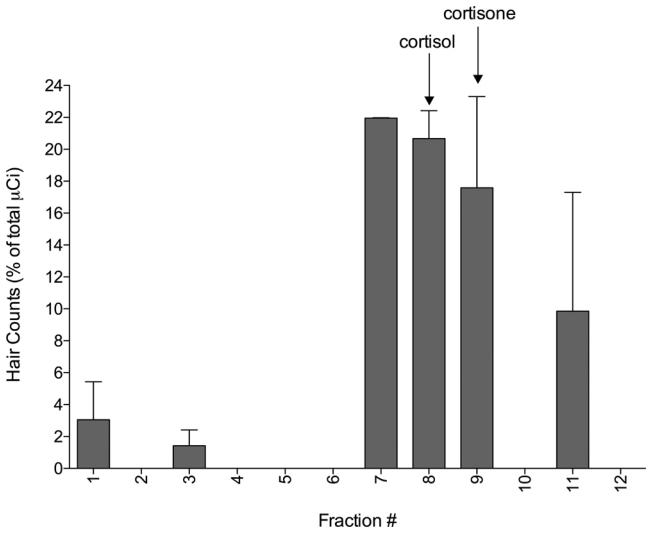

Fig. 4. Percent of total μCi measured in HPLC fractions from day 14 re-shave hair. Arrows indicate position of standard.

4. Discussion

This study is the first to directly show that cortisol in the blood is represented by cortisol in the hair, and therefore, contributes to the validation literature demonstrating that hair cortisol is a measure of central HPA axis activity. Specifically, this study has lent credence to the central pathway; that cortisol circulating in the bloodstream diffuses into dividing keratinocytes and/or melanocytes in hair follicle. The cortisol is incorporated into the hair matrix and moves with the hair shaft as it grows (Cone, 1996; Harkey, 1993).

Another proposed mechanism for incorporation of cortisol into hair is more indirect whereby cortisol from the blood is actively or passively diffused from body secretion (sweat, sebum) into dividing cells in hair follicle. Data from this study could also support such a mechanism. Since [^{3}H] was detected in the hair wash solution, it is possible that this was coming from secretions by the sweat or sebum that deposited externally on the hair shaft. Data from radiolabel studies in humans demonstrated that IV administration of cortisol was found in the sweat and the sebum (Cook and Spector, 1964; Jenkins et al., 1969). Further studies to support this would be needed to rule out external contamination from urine and/or feces on the hair of the monkeys.

Human studies measuring hair cortisol segment the hair based on a universal standard of hair growth rate (1 cm/month) to measure hair cortisol that would have been circulating during a specific time. The results of this study would caution against using this practice. First, hair growth rate was variable among the monkeys and the variability mimicked that of published human scalp hair growth rates indicating that a universal standard of growth rate is oversimplified (Giovanoli-Jakubczak and Berg, 1974; Harkey, 1993). Second, in this study we were also able to test, if by using the knowledge of the monkey's individual hair growth rate, we could cut hair from a previously unshaved area and find similar levels of [^{3}H] to the re-grown patches. This approach was not successful suggesting that segmenting hair samples to represent specific windows in time is not appropriate. Even with knowledge of the individual growth rate of the hair, we were not able to detect radioactivity in cut hair.

A potential explanation for the new-shave finding has been alluded to in the literature; that hair cortisol may not be stationary in the shaft once it is incorporated. Indeed, studies in humans and bears have shown that a cutaneous local stressor, that is independent of the central HPA axis, leads to immediate changes in hair cortisol that can be detected in the shaft of the hair (Cattet et al., 2014; Sharpley et al., 2009, 2010). Inherent in these findings is the ability for cortisol to rapidly

travel from the site of production in the hair follicle which is 3–4 mm below the skin and into the hair shaft to be cut and measured only minutes after application of a local stressor. Taken together, the results of the studies by Cattet et al., and Sharpley et al., as well as the current data, suggests that the premise that hair is a static material in which hormone is deposited and remains as the hair grows, is not valid.

Another, complementary, explanation for the new-shave finding is due to the stage of the hair growth cycle. Hair grows in cycles with periods of new growth (anagen), transition (catagen) and quiescence (telogen) and incorporation of hormones into hair is thought to occur during only during anagen, when the follicle is connected to the central blood supply (Thom, 2016; Cone, 1996). In the re-shave hair, all of the hair that grew was in the anagen phase and therefore would have incorporated the radiolabel. In contrast, the new-shave hair was in various stages of the hair growth cycle and the proportion of hair in the anagen phase would have been diluted with those from other phases. It is important to note however, that researchers have been using segmented hair to isolate specific windows of hormone activity where the hair would be in various stages of the hair growth cycle. Both of the explanations for the results of the new-shave hair would cautious against using this technique.

Another major finding from this study is that a proportion of circulating [^{3}H]-cortisol was also incorporated in the hair as cortisone and other unknown metabolites of cortisol as seen in Fig. 4. The only other radio-label study that was conducted for hair cortisol was in guinea pigs where they found the form of [^{3}H] detected in hair was [^{3}H]-cortisone and no [^{3}H] was found in the fraction that eluted in the position of the cortisol standard (Keckeis et al., 2012). Therefore, in both the current radiolabel study and that by Keckeis et al., cortisol from the bloodstream was found in the hair as cortisone, but the proportion varied between species. There are two potential explanations for this finding. First, cortisone is less polar than cortisol and studies of drugs of abuse in hair demonstrated that incorporation of drug into the keratin matrix of the hair shaft is favored by low polarity, non-conjugated and unbound compounds (Gow et al., 2010; Sauvé et al., 2007), which suggests that serum cortisone is more readily incorporated into hair than serum cortisol. This also fits with previously published data in rhesus macaques (Kapoor et al., 2014, 2016) and humans (Raul et al., 2004). Both show that the ratio of cortisol to cortisone is lower in hair compared to blood. A second possible explanation, offered by Raul et al., is that since 11β-HSD2, which converts cortisol to cortisone, is located in the superficial cells in duct of eccrine sweat glands of the skin, it may play a role in converting circulating cortisol to cortisone immediately before hormone incorporation into hair (Raul et al., 2004). Further studies are required to determine the merit of either, or both, of these explanations.

The form of cortisol metabolites incorporated into hair has emerged to be more complex than previously anticipated. In addition to incorporation of radiolabeled cortisol and cortisone from the bloodstream, there were other metabolites of [^{3}H]-cortisol that were found in the hair. The unknown in fraction 7 appeared to be an especially important hair metabolite of circulating cortisol as it was measured in both monkeys and radioactivity levels were similar to cortisol and cortisone. A screening approach to determine the identity of these unknowns was utilized where cortisol metabolites that had previously been identified from studies in urine (Zhai et al., 2015) were run through the chromatography to determine if their elution profiles matched the position of any of the unknowns. This approach was not successful likely because, up to now, identification of cortisol metabolites has focused on those in the urine which is the major pathway for cortisol excretion. Further studies to separate and characterize the cortisol metabolites found in the hair using advanced chromatography and mass spectrometry techniques are currently on-going.

The overall aim of this study was to determine the biological relevance of cortisol in hair. We have shown that cortisol from the bloodstream is found in the hair as cortisol, cortisone and other metabolites. Another mechanism that may represent an important source of hair glucocorticoids is from local production of cortisol in the hair follicles. A seminal study by Ito (Ito et al., 2005) reported that all the machinery required for synthesis and release of cortisol was found within skin cells, including hair follicles and referred to as the 'peripheral' HPA axis (Sharpley et al., 2009). The function of this axis is hypothesized to regulate the cutaneous response to local stressors such as solar radiation, infection, trauma and chemical irritants. The ability of the hair follicle itself to produce cortisol raises the possibility that local stressors to the skin and/or hair can trigger production of cortisol (Slominski et al., 2013). It is not clear whether this peripheral pathway is coupled to the central pathway and if so, to what degree. A study by Grass et al., demonstrated that a treadmill stressor that increased salivary cortisol levels was not detectable in the hair by 15 min, suggesting that the central and peripheral pathways are not linked (Grass et al., 2015). In the current study we did not address the potential contribution of the peripheral axis and therefore further work is required to determine precisely how to utilize the data generated. Once we have determined all the sources of cortisol reflected in the hair and the metabolites that represent circulating cortisol, algorithms can be created to accurately calculate the hair glucocorticoids that reflect circulating levels.

Radiolabel studies are the gold standard to determine metabolism of a compound (Penner et al., 2012). By utilizing a monkey model, we were able to conduct this crucial validation study which would have been problematic to carry out directly with humans. To accurately mimic the biological condition, a continuous infusion experiment of [^{3}H]-cortisol would have been more appropriate since endogenous cortisol is continually available for incorporation into hair. This was not feasible, however, due to the amount of radiation exposure that would be required for such an experiment. We opted to conduct this pulse-labeling study which generated important data toward understand the incorporation of circulating cortisol into hair. It has long been known that the rhesus monkey is an ideal model for cortisol metabolism in humans (Pal, 1979). In line with this, it has been shown that IV administered cortisol can been detected in the urine by 4 h after administration (Jung et al., 2014) which fits with the data from this study showing that most of the radiolabeled cortisol was found in the urine the day after injection. Further, the majority of the [^{3}H]-cortisol was excreted via urine compared to feces as shown in other studies of nonhuman primates (Bahr et al., 2000) and humans (Beisel et al., 1964). For this study, we decided not to collect blood samples to monitor the time-course of [^{3}H] disappearance from circulation since that would have required multiple administrations of sedation and immobilization for the monkeys.

The only other radiolabel validation of hair cortisol was conducted in a guinea pig, and this study showed the cortisol in the bloodstream was incorporated as cortisone only (Keckeis et al., 2012). The study design may have played a role in the Keckeis study, however, as the hair was collected the day before the third and final radiolabel cortisol injection. Nonetheless, this difference between the findings in the guinea pig and the rhesus monkey highlight the importance of using a model species that is closely related to humans in order to be able to translate the data.

There are some limitations on the study. First, only adult males were used, so there may be some differential effects of cortisol incorporation into the hair by age or sex. Second, this study did not permit us to determine the proportion of cortisol, cortisone and other metabolites that went into the hair from circulation. This information is required to develop the algorithms to use the metabolite data. Finally, the number of monkeys for the final analysis was low, but critical information was garnered from experiment 1 to determine the correct levels of radiolabel and time of hair collection for experiment 2, while in experiment 2, two monkeys did not regrow hair even up to 2 months after the initial shave.

Overall, this study has demonstrated that circulating levels of cortisol are represented by cortisol/cortisone measured in the hair. While

this study has provided support for the central pathway HPA axis, it has also raised many questions that speak to the complexity of cortisol incorporation into hair and nature of its disposition.

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