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Progesterone metabolism in human saliva in vitro

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Abstract

Human salivary glands are known to be able to metabolize progesterone as well as other steroid hormones. The rate of progesterone metabolism in the salivary glands is so low that it is not thought to affect salivary progesterone concentrations. On the other hand it is usually recommended that saliva should be frozen quickly after the collection to prevent any kind of metabolism in saliva. When saliva is collected at home e.g. delayed freezing or partial thawing during to transport to laboratory may create circumstances where progesterone metabolism may occur. However, it is not known to which extent progesterone metabolism continues in saliva and whether this continued metabolism of progesterone affects salivary hormone levels.

Paraffin-stimulated salivary samples were collected from female ($N = 6$) and male ($N = 6$) dental students and perimenopausal women ($N = 8$). The salivary samples were incubated with ^{14}C-progesterone for 2 h at 37°C in a shaking water bath. Metabolites were analyzed using thin-layer chromatography and autoradiography and quantified by liquid scintillation counting.

Human saliva was found to be able to metabolize progesterone, but its metabolic activity was very low, 9.3 and 6.8 pmol/ml/h in young adults and perimenopausal women, respectively. Metabolic activity was higher in whole saliva than in the corresponding activities of the supernatant or sonicated fraction of the same saliva. The supernatant fraction, which was thought to be mainly representative of glandular saliva, was metabolically least active. The polar metabolites of progesterone predominated in all incubations. The metabolic activity of saliva is probably mainly due to its cellular content and the contribution of this activity to salivary progesterone concentrations is not significant.

Keywords: Progesterone; Metabolism; Saliva

1. Introduction

The physiology of the human mouth is controlled by sex steroid hormones, and many of its tissues are involved in the metabolism of these hormones. Oral mucosa and gingival tissue are exposed to steroid hormones by blood circulation and saliva. Human oral mucosa and gingival tissue also contain receptors at least for androgens [1,2]. The main metabolite of progesterone in healthy and inflamed human gingiva is 20α-hydroxy-4-pregnen-3-one [3]. The metabolism of the steroid hormones increases significantly in inflamed human gingiva [3–6]. Sirigu et al. [7] showed that human salivary gland tissue contains steroid hormone metabolizing enzymes. This metabolic activity was later shown to be low and mainly catabolic [8]. Progesterone was mainly converted to 20α-hydroxy-4-pregnen-3-one as it is in gingival tissue.

Salivary progesterone measurements have been used to evaluate luteal progesterone secretion. The metabolism of progesterone in salivary glands is not thought to influence these salivary progesterone measurements significantly, because steroids passage rapidly from blood through the salivary glands into saliva [8]. In addition, good correlations have been shown between serum (total as well as free) progesterone and salivary

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progesterone levels [9,10]. On the other hand, the ratio between salivary and free serum progesterone is found to vary during the menstrual cycle [10,11], which may be due to metabolism occuring in the salivary glands.

The human mouth is a complex ecosystem with more than 300 bacterial species. One ml of human saliva may contain more than 10^8 microorganisms. Some bacterial species are known to be able to synthesize steroid metabolizing enzymes needed for steroid synthesis and catabolism. Most of these microorganisms belongs to intestinal flora, but some of them are also found in the mouth. Previous studies with two oral microorganisms, *Streptococcus mutans* (strain Ingbritt) and *Bacillus cereus* (strain Socransky 67), have shown that there is a quantitative and qualitative difference in progesterone metabolism between these microorganisms. The metabolic activity of *Bacillus cereus* is about twenty times higher than that of *Streptococcus mutans* [12]. Both strains were found to contain 20α- and 5α-hydroxysteroid dehydrogenases and steroid hydroxylases. In addition, *Bacillus cereus* contained 20β-hydroxysteroid dehydrogenase activity. The microenvironments of different parts of the mouth differ in steroid supply, pH and redox conditions, which may partly explain the variations in progesterone metabolism.

Whole saliva contains several constituents which may be involved in steroid metabolism, such as pure (i.e. glandular) saliva, leukocytes, microorganisms, exfoliated epithelial cells and gingival crevicular fluid, an exudate from plasma rich in steroid hormones. Crevicular fluid enters the oral cavity via the gingival sulcus and its flow to the oral cavity is increased when the gingiva is inflamed. ElAttar [13] used antibiotics in saliva incubations to reduce any effect of microorganisms on estrone and progesterone metabolism. He found that progesterone in the saliva of those with clinically healthy gingiva was not metabolized, whereas progesterone was converted to 5α- and 5β-pregnane-3,20-diones in saliva of those with inflamed gingiva. The metabolism seemed to be due to oral leukocytes, which show increased numbers in chronic periodontal disease. The aim of the present study was to evaluate how actively saliva metabolizes progesterone in vitro and to find out the source of this metabolic activity might be.

2. Materials and methods

2.1. Salivary samples

Twelve volunteer dental students (six men, six women) and eight perimenopausal women all in good oral health, participated in the study. Paraffin-stimulated whole saliva was collected after 30 s prestimulation. The saliva samples of the students was divided to two 5 ml parts. The first was used immediately for incubation with progesterone. The second part was centrifuged ($18{,}800 \times g$, 10 min, 4°C), and the supernatant was used immediately for incubation with progesterone. The sediment was frozen (−70°C). Within two months, the samples were thawed and sonicated for 2 min in sucrose buffer in a test tube kept in ice. The sonicated sediment was diluted to 5 ml (the original salivary volume) with sucrose buffer and used for incubation with progesterone. In perimenopausal women only whole saliva was used for incubation. The salivary flow rate (mean and S.D.) was 1.7(0.9) ml/min in students and 1.2 (0.5) ml/min in perimenopausal women.

2.2. Incubations

^{14}C-Progesterone with a specific activity of 51 Ci/mol (New England Nuclear, Boston, USA) was purified by using bidimensional thin-layer chromatography before use. Most of the reference steroids were donated by Professor D.N. Kirk, M.R.C. Steroid Reference Collection, Westfield College, London. Buffers (0.067 M KH_2 PO_4–$NaHPO_4$, pH 7.4) containing 1.0 mM EDTA with 0.25 mol/l sucrose (sucrose buffer) or without it (phosphate buffer) were used. The method of steroid incubation, extraction and thin-layer chromatography has been developed by Nienstedt [14,15] and Harri et al. [16,17]. For a detailed description see Laine and Ojanotko-Harri [18,19]. The incubation mixture contained 5 ml of saliva or supernatant or sonificate and 4 ml phosphate buffer containing an NADPH regenerating system consisting of 2.3 μmol NADP and 18.8 μmol glucose 6-phosphate and 3 units of glucose 6-phosphate dehydrogenase. The reaction was started by adding 2–3 nmol of radioactive progesterone dissolved in 1 ml phosphate buffer to the incubation mixture, which was incubated at 37°C for 2 h in a water bath. Blank incubation was done with the buffer without the saliva in every incubation. The reaction was stopped by placing the tubes on ice and adding, with shaking, 4 ml methylacetate to the tubes.

2.3. Extraction

The methyl acetate phase (upper phase) was removed after centrifugation ($1000 \times g$ for 20 min, 4°C). Extraction with methylacetate was repeated three times. The combined phases were evaporated under a stream of nitrogen at 50°C. The combined lower aqueous phases (containing possible water-soluble steroid conjugates) were mixed with ethanol, evaporated at 100–105°C and placed in a β-spectrometer for radioactivity counting.

After evaporation the upper or methyl acetate phase

Table 1
The quantity (mean ± S.D.) of the metabolites of progesterone (pmol/ml × h) in human saliva

Metabolite	Perimenopausal women	Young adults		
	whole saliva (N = 8)	whole saliva (N = 12)	supernatant fraction (N = 12)	sonicated fraction (N = 12)
More polar compounds	2.7 ± 1.0	4.3 ± 4.3	0.7 ± 0.8	2.3 ± 1.3
17α-Hydroxy-4-pregnene-3,20-dione	0.3 ± 0.6	1.1 ± 0.8	0.2 ± 0.2	0.1 ± 0.1
20α-Hydroxy-4-pregnen-3-one	0.8 ± 0.7	0.4 ± 0.6	0.3 ± 0.6	0.2 ± 0.2
5α-Pregnane-3,20-dione	0.8 ± 0.6	0.8 ± 0.6	0.2 ± 0.2	0.1 ± 0.2
5β-Pregnane-3,20-dione	1.3 ± 1.6	1.4 ± 1.3		0.1 ± 0.1
20β-Hydroxy-5α-pregnan-3-one[a] / 3α-Hydroxy-5β-pregnan-20-one	0.3 ± 0.3	0.4 ± 0.6		
3α-Hydroxy-5α-pregnan-20-one	0.2 ± 0.2	0.3 ± 0.3		
3β-Hydroxy-5α-pregnan-20-one	0.2 ± 0.1	0.3 ± 0.4	0.1 ± 0.2	
5β-Pregnane-3α,20α-diol[a] / 5β-Pregnane-3α,20β-diol		0.3 ± 0.3		
Total metabolism (sum of all metabolites)	6.8 ± 2.4	9.3 ± 6.2	1.9 ± 1.7	2.8 ± 1.7

[a] Quantified together because of the partial overlapping of chromatographic spots .

(possible lipid-soluble compounds) to 0.3 ml, 1.7. ml of methanol and 0.8 ml of hexane were added. After mixing and centrifugation at 1000 × g for 20 min two phases was separated: the upper (hexane) phase contained possible lipoidal steroids and the lower (methanol) phase containing free steroids.

The radioactivity of the three phases (lipoidal metabolites, steroid conjugates and free steroid fraction) was determined by liquid scintillation counting. Most radioactivity was found in the free steroid fraction (more than 93% of total radioactivity). This was the only fraction that was examined further and used for bidimensional thin-layer chromatography.

2.4. Thin-layer chromatography

The free steroid fraction together with reference steroids were pipetted onto silica gel plates, and the plates were run with bidimensional thin-layer chromatography. The solvent systems were dichlormethane-methyl acetate (9:1, v/v) for the first dimension and hexanol-hexane (1:3, v/v) for the second dimension. Autoradiograms of the plates were made on X-ray films with an exposure time of about 30 days. Then the reference steroids that had been added to the plates were visualized by staining the plates with ethanol-acetic anhydride-sulphuric acid. The stained spots were separated by cutting, and radioactivity was determined by liquid scintillation counting. Each radioactive spot represented some metabolite. Four minor metabolites, 20β-hydroxy-5α-pregnan-3-one and 3α-hydroxy-5β-pregnan-20-one as well as 5β-pregnane-3α, 20α-diol and 5β-pregnane-3α,20β-diol separated incompletely and were quantified together. Most of the more polar metabolites remained unidentified because reference steroids were not available.

2.5. Calculations

The concentrations of formed progesterone metabolites were determined as pmol/h/ml of saliva/supernatant/sonicate. The sum of the metabolite concentrations was defined as total metabolism per hour.

Since no sex differences were found in the metabolism in young adults, the female and male groups were analyzed together.

The statistical analysis of the results of total metabolism was carried out using Wilcoxon's test for mean values.

3. Results

Table 1 shows the metabolites and total metabolism of progesterone. The progesterone metabolizing activity of whole saliva was higher than the corresponding activities in supernatant or in sonicated fraction of same saliva. The total metabolism of progesterone was low. The saliva of young adults was metabolically more active than that of perimenopausal women, and continued metabolism of 3α-hydroxy-5β-pregnan-20-one to 5β-pregnane-3α,20α-diol/ 5β-pregnane-3α,20β-diol could only be detected in the saliva of young adults, but no statistically significant differences were found between the groups.

On the basis of their chromatographic mobility it was concluded that several of the unidentified metabolites were polar, with more than two functional groups (hydroxyl and/or keto). These polar metabolites, however, are not necessarily hydroxylated [14,15]. This group of metabolites remained unidentified because corresponding reference steroids were not available and referred as more polar metabolites. In several incubations with whole saliva, supernatant and

sonicated fraction of saliva one more polar metabolite was separated completely from other more polar metabolites and matched with authentic 11α-hydroxy-4-pregnene-3,20-dione.

The supernatant fraction, which was thought to be mainly representative of glandular saliva, was metabolically the least active. The sonicated fraction was thought to be mainly representative of the cellular fraction of the saliva, which mainly consists of microorganisms but also contains exfoliated cells and leukocytes. In this fraction over 80% of the metabolites were more polar ones.

The metabolites found after saliva incubations suggest that the following enzymes are involved in progesterone metabolism: 5α- and 5β-steroid hydrogenase, 3α-, 3β-, 20α- and 20β-hydroxysteroid dehydrogenase activities. Saliva also contains 11α- and 17α-hydroxylases and probably other hydroxylases (due to the polar metabolites).

4. Discussion

The in vitro metabolism of progesterone by human saliva and its components was low. Since the specific gravity of saliva is about 1.0 (i.e. 1 ml of saliva weight about 1 g), it can concluded from this study and from a previous study of our laboratory [8] that progesterone metabolism in saliva is about 50–240 times lower than that in parotid and submandibular salivary gland homogenates. The unidentified, more polar metabolites constituted about half of the metabolites in whole saliva and predominated in the sonicated fraction. One of the more polar metabolites, 11α-hydroxy-4-pregnene-3,20-dione, is a typical metabolite of several bacterial species [20] and not found in human salivary glands. Small amounts of 17α-hydroxy-4-pregnen-3,20-dione (17α-hydroxyprogesterone), which is an intermediate for estrogen and androgen biosynthesis, were found. Progesterone was also converted to 20α-hydroxy-4-pregnen-3-one. This metabolite has some progestational activity and is the main metabolite in the human salivary glands [8] and human gingiva [3]. All other identified metabolites were 5α- or 5β-reduced products of progesterone. Some steroid metabolizing enzymes are in a soluble form in the salivary glands and in gingiva. It is not yet known whether these soluble enzymes are secreted from the salivary glands into saliva. The metabolic activity in the supernatant fraction was lowest. Although this fraction mainly represents glandular saliva, it also contains soluble enzymes from cells either secreted or released after cell lysis. Due to the more polar metabolites found in whole saliva and its subfractions, it is probable that the salivary metabolic activity mainly originates from the cellular fraction of whole saliva. This fraction mainly consists of microorganisms, but exfoliated cells and leucocytes also occur. As shown before, the metabolic patterns of oral microorganisms vary greatly from strain to strain that the microbial source of metabolic activity in the sonicated fraction could not be determined.

The difference in collection techniques (unstimulated/ paraffin-stimulated saliva) does not influence the concentration of progesterone in saliva since steroid hormone concentrations in saliva are independent of the salivary flow rate [9,21]. When salivary samples are collected for hormone measurements, the collection is usually carried out without stimulation to avoid contamination by other steroid hormones originating from the blood or gingival crevicular fluid. We used paraffin-stimulated saliva because of the large volume needed for incubations. Our subjects had good oral health which minimized any contamination by the blood or crevicular fluid. Paraffin-chewing detaches bacteria from tooth and other surfaces. In this sense stimulated saliva may be metabolically even more active than unstimulated saliva. The endogenous progesterone could not significantly affect the results since the final progesterone concentration (200–300 nmol/l) in incubation mixture exceeded e.g. the average luteal peak level of salivary progesterone [22] several hundred times.

In conclusion, progesterone was metabolized by human whole saliva but this metabolical activity is low compared to that of salivary glands. The metabolic activity in saliva is probably due to its cellular content. Usually when saliva is collected for analysis, it is frozen as quickly as possible. Many salivary constituents, especially proteins, are degraded by enzymes originating from salivary cellular components or salivary glands. When saliva is collected at home for progesterone measurements, it is possible that some progesterone metabolism may occur as a consequence of delayed freezing of saliva or short-time thawing of the saliva during transport to laboratory [23]. The metabolic activity is, however, so low even in optimal incubation conditions that its effect on salivary progestrone concentrations in above mentioned circumtances is probably unimportant. On the whole, other factors as bleeding due to gingivitis or periodontitis are much more important sources of error in salivary progesterone measurements than salivary progesterone metabolism itself.

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